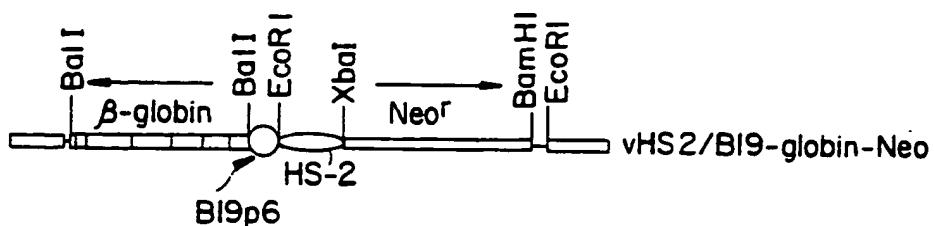
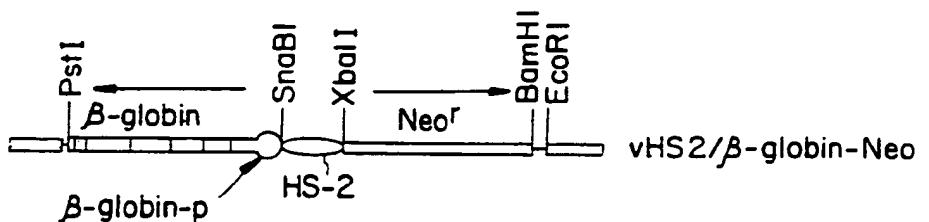




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :  C12N 15/86, 15/12, A61K 48/00	A1	(11) International Publication Number: WO 93/09239  (43) International Publication Date: 13 May 1993 (13.05.93)
(21) International Application Number: PCT/US92/09769		(81) Designated States: AU, CA, HU, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).
(22) International Filing Date: 6 November 1992 (06.11.92)		
(30) Priority data: 789,917 8 November 1991 (08.11.91) US		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(71) Applicant: RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; 6840 East Broadway Boulevard, Tucson, AZ 85710 (US).		
(72) Inventor: SRIVASTAVA, Arun ; 975 West Walnut Street, Indianapolis, IN 46202 (US).		
(74) Agents: SCOTT, Anthony, C. et al.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).		

## (54) Title: ADENO-ASSOCIATED VIRUS-2 BASAL VECTORS



## (57) Abstract

Gene therapy involves the transfer and stable insertion of new genetic information into cells. The present invention is directed to safe vectors for gene therapy and thus provides hybrid parvovirus vectors which are capable of site-specific integration into a mammalian chromosome without substantial cytotoxicity, and which direct erythroid cell-specific expression of heterologous genes. The hybrid vector is useful in gene therapy, particularly in the treatment of hemoglobinopathies and other hematopoietic diseases, and in conferring cell-specific multidrug resistance. A method of delivery of constitutive levels of a pharmaceutical product and a method of producing a recombinant protein are also provided.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BC	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CC	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TC	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam

1

## ADENO-ASSOCIATED VIRUS-2 BASAL VECTORS

Gene therapy involves the transfer and stable insertion of new genetic information into cells. The present invention is directed to a safe vector for gene therapy and provides hybrid parvovirus vectors which are capable of site-specific integration into a mammalian chromosome without substantial cytotoxicity, and which can direct cell-specific expression of a desired gene product. The hybrid vectors are useful in gene therapy, particularly in the treatment of hemoglobinopathies. A method of delivery of a pharmaceutical product is also provided. The present invention also provides a method of conferring cell-specific multidrug resistance.

The therapeutic treatment of diseases and disorders by gene therapy involves the transfer and stable insertion of new genetic information into cells. The correction of a genetic defect by re-introduction of the normal allele of a gene encoding the desired function has demonstrated that this concept is clinically feasible [Rosenberg *et al.* (1990) New Eng. J. Med., 323, 570].

Hematopoietic stem cells or pluripotent progenitor cells are particularly useful for gene therapy studies since, although they are somatic cells, they differentiate to produce all the lineages of blood cells. Hence, the introduction of a foreign gene into a stem or progenitor cell results in the production of various lineages which can potentially express the foreign gene or alter control of native gene products. The introduction of a foreign gene into a progenitor cell or any other appropriate cell requires a method of

-2-

1 gene transfer to integrate the foreign gene into the  
cellular genome. Although a variety of physical and  
chemical methods have been developed for introducing  
exogenous DNA into eukaryotic cells, viruses have  
5 generally been proven to be much more efficient for this  
purpose. Several DNA-containing viruses such as  
parvoviruses, adenoviruses, herpesviruses and  
poxviruses, and RNA-containing viruses, such as  
retroviruses, have been used to develop eukaryotic  
10 cloning and expression vectors. The fundamental problem  
with retroviruses is that they are either the etiologic  
agents of, or are intimately associated with,  
malignancy. Retroviruses integrate randomly into the  
cellular genome, and thus may activate cellular proto-  
15 oncogenes or may disrupt sequences critical to cell  
function. Accordingly, the use of retroviral vectors in  
gene transfer presents a problem in that there is a  
finite chance that such vectors may induce neoplasia.  
Thus, a need exists for additional and improved vectors  
20 for gene transfer.

Whereas retroviruses are frequently the etiologic  
agents of malignant disorders, parvoviruses constitute  
the sole group of DNA-containing viruses that have not  
yet been associated with any malignant disease.  
25 Although parvoviruses are frequently pathogenic in  
animals, a parvovirus of human origin, the adeno-  
associated virus 2 (AAV), has so far not been associated  
with any known human disease, even though up to 90% of  
the human population has been exposed to AAV.  
30 [Blacklow, N. R. (1988) in: Parvoviruses and Human  
Disease, CRC Press, Boca Raton]. In addition, most  
retroviruses used for gene transfer are of murine

-3-

1 origin, while AAV, a human virus, is physiologically  
more relevant for gene transfer in humans. Moreover,  
retroviruses are susceptible to inactivation by heat and  
organic solvents, whereas AAV is heat stable, extremely  
5 resistant to lipid solvents, and stable between pH 3.0  
and 9.0. Thus as vehicles for gene transfer,  
parvoviruses provide many advantages over retroviruses.

Recombinant retroviruses have low viral titers  
( $10^5$ - $10^6$  virions/ml) (Rosenberg) in contrast to the high  
10 titers of recombinant AAV ( $10^8$ - $10^9$  virions/ml)  
[Srivastava *et al.* (1990) Blood 76, 1997].  
Consequently, it is generally not possible to achieve an  
infection efficiency with recombinant retrovirus beyond  
15 10-50% of the target cell population, with successful  
infection requiring actively replicating cells. In  
contrast, a 70% infection efficiency has been reported  
for a recombinant AAV [Samulski *et al.* (1989) J. Virol.  
63, 3822], and it is possible to achieve a 100%  
infectivity of target cells with wild-type AAV [Nahreini  
20 *et al.* (1989) Intervirology 30, 74]. Furthermore, even  
though recombinant retroviral vectors have been rendered  
replication-incompetent, there remains a low probability  
of recombination between the vector and endogenous  
retroviral sequences. In contrast, 60-90% of the  
25 population is sero-positive for human parvoviruses, and  
no endogenous viral sequences have yet to be detected in  
volunteer donors. In recombinant AAV vectors, all of  
the AAV coding sequences have, nonetheless, been  
deleted.  
30 Perhaps the most significant advantages of AAV-  
based vectors are that they mediate integration into the  
host chromosomal DNA in a site-specific and stable

-4-

1 manner. Retroviral genomes, following reverse  
transcription, undergo integration into the host  
chromosomal DNA with a totally random integration  
pattern. AAV establishes a latent infection which is  
5 site-specific. The integration site has been mapped to  
human chromosome 19. (Kotin et al. (1990) Proc. Natl.  
Acad. Sci. USA 87, 2211). It has therefore become  
feasible to accomplish site-specific delivery of  
exogenous DNA into mammalian cells. While retroviral  
10 vectors mediate integration of non-viral sequences into  
the host chromosome, the integration pattern is not  
always stable. Frequently the integrated retroviral  
provirus is excised from the cell. AAV, on the other  
hand, establishes a stable integration.

15 Despite the potential advantages outlined above,  
the parvovirus-based vectors suffer from one limitation,  
and that is the size of a DNA sequence that can be  
packaged into the mature virions. For example, whereas  
up to 8.0 - 9.0 kilobase pair (kbp) DNA fragments can be  
20 packaged into retroviral vectors, a maximum of about 5.0  
kbp DNA can be packaged into AAV. This size limitation,  
however, does not preclude the cloning and packaging of  
most cDNA molecules.

Thus parvovirus-based vectors offer a useful  
25 alternative to retroviral vectors for gene therapy in  
humans. While AAV-based vectors allow stable, site-  
specific integration of transferred genes, the  
indiscriminate expression of the transferred gene in all  
cell lineages presents significant problems. Thus, a  
30 need exists for AAV vectors which effect tissue-specific  
expression of the transferred gene. In accordance with  
the present invention, one method, for example, to solve

-5-

1 this problem is by a combination of the features of AAV  
and another human parvovirus, B19.

While AAV causes no known disease, B19 is known  
5 to be the etiologic agent of a variety of clinical  
disorders in humans. B19 is the causative agent of  
transient aplastic crises associated with various  
hemolytic anemias, erythema infectiosum or the "fifth  
disease", post-infection polyarthralgia and  
thrombocytopenia in adults, and some cases of chronic  
10 bone marrow failure and hydrops fetalis.

AAV is dependent on a helper virus, such as  
adenovirus, herpesvirus, or vaccinia virus, for optimal  
replication. In the absence of a helper virus, AAV  
establishes a latent infection in which the viral genome  
15 integrates into chromosomal DNA site-specifically. B19,  
on the other hand, is an autonomously replicating virus  
that is known to replicate only in human hematopoietic  
cells in the erythroid lineage. Both AAV and B19  
contain linear, single-stranded DNA genomes, but their  
20 genomes show no homology at the nucleotide sequence  
level. The nucleotide sequences of both genomes are  
known. [Lusby *et al.* (1980) *J. Virol.* **34**, 402,  
Srivastava *et al.* (1983) *J. Virol.* **45**, 555; Shade *et al.*  
25 (1986) *J. Virol.* **58**, 921]. The AAV genome contains  
inverted terminal repeats (ITRs) of 145 nucleotides, 125  
nucleotides of which form a palindromic hairpin that  
plays a critical role during AAV DNA replication. The  
sequences of the ITRs are shown in Fig. 1 and as SEQ ID  
NO:1. In latently infected cells, the termini of AAV  
30 are at the junction of the cellular sequences and thus  
the termini also facilitate integration and rescue.

-6-

1        The remarkable features of the two human  
parvoviruses can be combined, for example, in an AAV-B19  
hybrid vector, to provide vectors in accordance with the  
present invention. The vectors of this invention are  
5        particularly useful for gene transfer in bone marrow  
cells and other hematopoietic cells. These hybrid viral  
vectors mediate site-specific integration as well as  
tissue-specific expression of heterologous genes in  
hematopoietic cells.

10      The present invention is directed to hybrid  
parvovirus vectors capable of site-specific integration  
into a mammalian chromosome without substantial  
cytotoxicity, and which can direct tissue-specific  
expression of a heterologous gene, i.e. a non-parvovirus  
15      gene. More particularly, the present invention provides  
vectors comprising two inverted terminal repeats of  
adeno-associated virus 2 and at least one genetic  
cassette comprising a promoter capable of effecting  
cell-specific expression operably linked to a  
20      heterologous gene wherein the cassette resides between  
the two inverted terminal repeats. In a preferred  
embodiment, the promoter is the p6 promoter of B19  
parvovirus and directs erythroid cell-specific  
expression of the heterologous gene.

25      In another aspect of this invention, host cells  
transduced by the hybrid vectors of the present  
invention are provided.

Another aspect of the present invention provides  
a method of treatment for hematopoietic diseases, in  
30      particular hemoglobinopathies, by transducing  
hematopoietic stem or progenitor cells with a vector of  
a present invention and introducing the transduced cells

-7-

1 into a patient, where the heterologous gene is  
expressed.

5 A further aspect of this invention provides a method for delivery of a pharmaceutical product in a mammal by transducing hematopoietic stem or progenitor cells with a hybrid vector of the present invention and introducing the transduced cells into the mammal. The heterologous gene is expressed, and the mature red blood cell provides a vehicle for delivery of the heterologous gene product throughout the bloodstream or to the liver or spleen.

10 Yet another aspect of the present invention provides a method of conferring cell-specific multidrug resistance by transducing cells with a hybrid vector of the present invention in which the heterologous gene is a multidrug resistance gene, and introducing the transduced cells into a mammal. In a preferred embodiment, the hybrid vector contains the B19p6 promoter, and thus the multidrug resistance phenotype is conferred to erythroid cells.

15 As used herein, transduction refers to a process by which cells take up foreign DNA and integrate that foreign DNA into their chromosomes. Transduction can be accomplished, for example, by transfection, which refers to various techniques described hereinbelow by which cells take up DNA, or infection, by which viruses are used to transfer DNA into cells.

20 Fig. 1 depicts the nucleotide sequence of an ITR of the AAV 2 genome.

25 Fig. 2 depicts the nucleotide sequence of B19 from nucleotide number 200 to nucleotide number 424 as numbered by Shade et al. (1986).

-8-

1       Fig. 3 diagrams the construction of a hybrid  
vector of the present invention.

5       Fig. 4 demonstrates the site-specific integration  
of the wild-type AAV genome into normal human diploid  
chromosomal DNA by Southern blot analysis.

10      Fig. 5 demonstrates the site-specific integration  
of the recombinant AAV genome into normal human bone  
marrow cells by Southern blot analysis.

15      Fig. 6 is a diagram of the construction of  
recombinant plasmids pWP-7A (Panel A) and pWP-19 (Panel  
B).

20      Fig. 7 is a diagram of the construction of  
recombinant plasmid pWN-1.

25      Fig. 8 is a diagram of the construction of  
recombinant plasmids pWP-21 and pWP-22 (Panel A) and  
pWP-16 and pWP-17 (Panel B).

30      Fig. 9 demonstrates Southern blot analysis of  
rescue and replication of the recombinant neo<sup>r</sup> gene in  
human cells. Panel A: Rescue from plasmid pWP-8A;  
35     Panel B: Rescue from plasmid pWP-21; Panel C: Rescue  
from plasmid pWP-22; Panel D: Rescue from plasmid pWP-  
16; Panel E: Rescue from plasmid pWP-17. Recombinant  
plasmids were transfected separately in adenovirus-  
infected human KB cells (Lanes 1, 3, 5, 7, 9), or co-  
transfected with pAAV/Ad helper plasmid (Lanes 2, 4, 6,  
8, 10). m and d denote the monomeric and dimeric forms,  
respectively, of the recombinant AAV DNA replicative  
intermediates.

35      Fig. 10 is a diagram of the construction of a  
hybrid vector in which the neomycin resistance (Neo<sup>r</sup>)  
gene is under the control of the B19p6 promoter.

-9-

1        Fig. 11 is a graph depicting cell viability after  
AAV-mediated transfer of Neo<sup>r</sup> to human hematopoietic  
stem cells. Panel A illustrates Neo<sup>r</sup> gene expression  
under the control of the TK promoter. Panel B  
5        illustrates Neo<sup>r</sup> gene expression under the control of  
the B19p6 promoter.

10      Fig. 12 depicts recombinant AAV vectors of the  
present invention containing selectable genes under the  
control of B19p6 promoter and a human erythroid-specific  
enhancer (HS-2).

Fig. 13 depicts the recombinant AAV vectors of  
the present invention which express the human β-globin  
gene and the Neo<sup>r</sup> gene.

15      Fig. 14 provides a Souther blot of DNA isolated  
from vHS2/β-globin-Neo infected K562 cells.

Fig. 15 provides a Northern blot of RNA from K562  
cells infected with vHS2/β-globin-Neo and vHS2/B19-  
globin-Neo.

20      The present invention relates to hybrid  
parvovirus vectors which comprise a pair of AAV inverted  
terminal repeats (ITRs) which flank at least one  
cassette containing a promoter which directs cell-  
specific expression operably linked to a heterologous  
gene. Heterologous in this context refers to any  
25      nucleotide sequence or gene which is not native to the  
AAV or B19 parvovirus. In accordance with the present  
invention, AAV and B19 coding regions have been deleted,  
resulting in a safe, noncytotoxic vector.  
Representative heterologous genes are described  
30      hereinbelow. The AAV ITRs, or modifications thereof,  
confer infectivity and site-specific integration, but  
not cytotoxicity, and the promoter directs cell-specific

-10-

1 expression and preferably erythroid cell expression  
using the p6 promoter of B19 parvovirus. The hybrid  
vectors of the present invention thus provide DNA  
molecules which are capable of integration into a  
5 mammalian chromosome without substantial toxicity.  
These hybrid vectors allow safe integration of the DNA  
into the cellular genome, since the portions of the DNA  
responsible for replication of the parvovirus have been  
deleted, and therefore these vectors cannot self  
10 replicate.

In accordance with the present invention, the  
hybrid vector comprises a first and second terminal  
repeat which flank a promoter linked to a heterologous  
gene. The terminal repeats can comprise all or part of  
15 the ITRs of AAV. The terminal repeats mediate stable  
integration of the DNA sequence into a specific site in  
a particular chromosome, e.g. human chromosome 19. The  
entire DNA sequence, including the ITRs, the promoter,  
and the heterologous gene can be integrated into the  
20 cellular genome.

The terminal repeats of the hybrid vector of the  
present invention can be obtained by restriction  
endonuclease digestion of AAV or a plasmid such as  
psub201, which contains a modified AAV genome [Samulski  
25 et al. (1987) J. Virol. 61, 3096], or by other methods  
known to the skilled artisan, including but not limited  
to chemical or enzymatic synthesis of the terminal  
repeats based upon the published sequence of AAV. The  
ordinarily skilled artisan can determine, by well-known  
30 methods such as deletion analysis, the minimum sequence  
or part of the AAV ITRs which is required to allow  
function, i.e. stable and site-specific integration.

-11-

1 The ordinarily skilled artisan can also determine which  
minor modifications of the sequence can be tolerated  
while maintaining the ability of the terminal repeats to  
direct stable, site-specific integration. Site-specific  
5 integration can be assessed, for example, by Southern  
blot analysis. DNA is isolated from cells transduced by  
the vectors of the present invention, digested with a  
variety of restriction enzymes, and analyzed on Southern  
blots with an AAV-specific probe. A single band of  
10 hybridization evidences site-specific integration.  
Other methods known to the skilled artisan, such as  
polymerase chain reaction (PCR) analysis of chromosomal  
DNA can be used to assess stable integration.

The vectors of the present invention contain a  
15 promoter which directs tissue-specific expression. For  
example, the wild-type parvovirus B19 has a limited host  
range and exhibits a remarkable tissue tropism for the  
erythroid elements of bone marrow. In a preferred  
embodiment, the hybrid vectors of the present invention  
20 utilize a transcriptional promoter of B19 to effect  
tissue-specific expression of heterologous sequences.  
In a more preferred embodiment the promoter is the p6  
promoter of B19, which is active in erythroid progenitor  
cells. The nucleotide sequence of B19 from nucleotide  
25 number 200 to nucleotide number 424 as numbered by Shade  
et al. (1986) contains the p6 promoter and is depicted  
in Fig. 2 and as SEQ ID NO:2.

The consensus promoter-like sequence TATATATA is  
present at nucleotide 320 in B19 (as numbered by Shade  
30 et al.) and thus transcription is likely to originate  
about 30 nucleotides downstream. It has been discovered  
in accordance with the present invention that B19

-12-

1 fragments containing these sequences direct expression  
that is specific for erythroid progenitor cells, and  
that deletion of B19 coding sequences downstream from  
the promoter prevents replication of B19. As explained  
5 above, one of ordinary skill in the art can determine  
the minimum sequence and modifications of the p6  
promoter which provide cell-specific, non-cytotoxic  
expression. This can be determined by infecting  
erythroid and non-erythroid cells with vectors  
10 containing the B19p6 promoter and assessing expression  
of the heterologous gene. The promoter sequence can be  
derived by restriction endonuclease digestion of B19 or  
a cloned B19 plasmid such as pYT103 and pYT107 [Cotmore  
15 et al. (1984) Science 226, 1161] or by any other methods  
known to the skilled artisan, including but not limited  
to chemical or enzymatic synthesis based upon the  
published sequence of B19. Other cell-specific  
promoters can be obtained by analogous methods, and the  
specificity of these promoters is determined by  
20 assessing expression in the appropriate cell type.

The promoter of the hybrid vector is operably  
linked to the heterologous gene. Any gene that can be  
transcribed in such a construction is contemplated by  
the present invention. In a preferred embodiment, the  
25 heterologous gene encodes a biologically functional  
protein, i.e. a polypeptide or protein which affects the  
cellular mechanism of a cell in which the biologically  
functional protein is expressed. For example, the  
biologically functional protein can be a protein which  
30 is essential for normal growth of the cell or for  
maintaining the health of a mammal. The biologically  
functional protein can also be a protein which improves

-13-

- 1      the health of a mammal by either supplying a missing protein, by providing increased quantities of a protein which is underproduced in the mammal or by providing a protein which inhibits or counteracts an undesired
- 5      molecule which may be present in the mammal. The biologically functional protein can also be a protein which is a useful protein for investigative studies for developing new gene therapies or for studying cellular mechanisms.
- 10     The biologically functional protein can be a protein which is essential for normal growth or repair of the human body. The biologically functional protein may also be one which is useful in fighting diseases such as cancer, atherosclerosis, sickle-cell anemia and
- 15     the thalassemias. Examples of such biologically functional proteins are hemoglobin ( $\alpha$ ,  $\beta$  or  $\gamma$ -globin), hematopoietic growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF),
- 20     granulocyte colony stimulating factor (G-CSF) and erythropoietin (EPO). Another example is tumor necrosis factor (TNF), which is a molecule that can be used to treat cancer, and in particular, tumors. The tumor suppressors p53 and retinoblastoma (RB) are also
- 25     contemplated. Various cytokines such as mast cell growth factor (MGS) and interleukins 1-11 are also proteins which are contemplated by the present invention. The biologically functional protein may also be a selectable marker for antibiotic resistance such as
- 30     a selectable marker for neomycin resistance in eukaryotes. Other types of selectable markers such as adenine phosphoribosyl transferase (APRT) in APRT-

-14-

1 deficient cells, or the firefly luciferase gene are also included. The heterologous genes encoding these proteins can be provided by any of a variety of methods, such as routine cloning procedures (Sambrook et al.),  
5 excision from a vector containing the gene of interest, or chemical or enzymatic synthesis based on published sequence information. In many instances the DNA encoding the protein of interest is commercially available.

10 The biologically functional protein can affect cellular mechanism by providing a new or altered function to a cell. For example, the heterologous gene can be a multidrug resistance gene (*mdr*) which encodes P-glycoprotein. P-glycoprotein is a cell membrane  
15 glycoprotein which affects intracellular drug accumulation and is responsible for the phenomenon of multidrug resistance.(for review, see Biedler [1992] Cancer 70 1799)

In another embodiment the heterologous gene can  
20 encode a non-biologically functional protein. For example, a hybrid gene comprising various domains and functions from a variety of sources can be designed and produced by recombinant technology or enzymatic or chemical synthesis.

25 In another preferred embodiment the heterologous gene is capable of being transcribed into an RNA molecule which is sufficiently complementary to hybridize to an mRNA or DNA of interest. Such an RNA molecule is hereinafter referred to as antisense RNA,  
30 and has utility in preventing or limiting the expression of overproduced, defective, or otherwise undesirable molecules. The vector of the present invention can

-15-

1 comprise, as the heterologous gene, a sequence encoding  
an antisense RNA which is sufficiently complementary to  
a target sequence such that it binds to the target  
sequence. For example, the target sequence can be part  
5 of the mRNA encoding a polypeptide such that it binds to  
and prevents translation of mRNA encoding the  
polypeptide. In another embodiment, the target sequence  
is a segment of a gene that is essential for  
transcription such that the antisense RNA binds the  
10 segment (e.g. a promoter or coding region) and prevents  
or limits transcription. Hence, the antisense RNA must  
be of sufficient length and complementarity to prevent  
translation of its target mRNA or transcription of its  
target DNA.

15 In a preferred embodiment the antisense RNA is a  
15mer and exhibits 100% complementarity to the target  
sequence. One of ordinary skill in the art can  
determine longer or shorter antisense molecules having  
sufficient complementarity to a target sequence such  
20 that the antisense molecule is capable of binding to the  
target and thereby inhibiting translation or  
transcription. The heterologous gene can be provided,  
for example, by chemical or enzymatic synthesis, or from  
commercial sources.

25 It is preferable that the length of the  
heterologous gene is such that the overall size of the  
hybrid vector is about 5 kilobases (kb), since the  
packaging limit of AAV virions is about 5 kb (Hermonat  
*et al.* (1984) Proc. Natl. Acad. Sci. USA 81, 6466).

30 The hybrid vectors of the present invention can  
be provided by inserting the heterologous gene and the  
cell-specific promoter between a pair of AAV-derived

-16-

1 terminal repeats. The combination of a promoter and  
heterologous gene is also referred to herein as a  
cassette. Thus, the invention provides a vector in  
which: 1) the terminal repeats mediate stable, site-  
5 specific integration into the cellular genome; and 2)  
the promoter mediates cell-specific expression of a  
heterologous gene, e.g. in erythroid cells, or the  
promoter mediates transcription of an antisense RNA or a  
sense RNA encoding a polypeptide of interest. The  
10 promoter sequence is operably linked to the heterologous  
gene in a manner to effect expression of the gene.  
Hence, the promoter sequence can be at either or both  
ends of the heterologous sequence or coding region.  
Furthermore, more than one promoter and heterologous  
15 gene can be present in one vector, i.e. there can be two  
or more cassettes between the ITRs. Accordingly, more  
than one heterologous gene can be expressed by one  
vector.

Standard techniques for the construction of such  
20 hybrid vectors are well-known to those of ordinary skill  
in the art and can be found in references such as  
Sambrook *et al.* (1989) in Molecular Cloning: A  
Laboratory Manual, Cold Spring Harbor, New York, or any  
of the myriad of laboratory manuals on recombinant DNA  
25 technology that are widely available. A variety of  
strategies are available for ligating fragments of DNA,  
the choice of which depends on the nature of the termini  
of the DNA fragments and can be readily determined by  
the skilled artisan.

30 It is further contemplated in accordance with the  
present invention to include in the hybrid vectors other  
nucleotide sequence elements which facilitate

-17-

1 integration of DNA into chromosomes, expression of the  
DNA, and cloning of the vector. For example, the  
presence of enhancers upstream of the promoter or  
terminators downstream of the coding region can  
5 facilitate expression. In another example, recent  
studies have identified a DNasel-hypersensitive site  
(HS-2) upstream of the human globin gene cluster that  
significantly enhances the erythroid-specific expression  
of the globin genes. [Tuan et al. (1985) Proc. Natl.  
10 Acad. Sci. USA 82, 6384]. In the hybrid vectors of the  
present invention, the presence of HS-2 upstream of the  
B19p6 promoter enhances tissue specific expression.

As described hereinabove, the vectors of the  
present invention can be constructed by a variety of  
15 well-known methods, and the order of the ligation of the  
elements can be varied. In a preferred embodiment the  
cell-specific promoter and heterologous gene are ligated  
together to provide a cassette which can be inserted  
between two AAV-ITRs. For example, to provide a  
20 cassette containing the B19p6 promoter and a  
heterologous gene, a fragment containing the p6-promoter  
is inserted into a pUC19 plasmid, after which the p6  
containing plasmid is linearized by restriction enzyme  
cleavage downstream of the p6 promoter. The  
25 heterologous gene is then inserted immediately  
downstream of the p6 promoter. A fragment containing  
both the p6 promoter and the heterologous gene is  
excised from the plasmid and inserted between the AAV-  
ITRs in an AAV plasmid from which the AAV coding regions  
30 have been deleted. The resulting plasmid comprises the  
p6 promoter and a heterologous gene flanked by a pair of  
AAV-ITRs. This construction is described more

-18-

1 specifically as follows and is diagrammed in Fig. 3. To  
generate a plasmid containing p6, a fragment containing  
the p6 promoter of B19 is isolated from B19 DNA or  
cloned B19 DNA [see, for example, Cotmore et al. (1984);  
5 Shade et al. (1986)]. In a preferred embodiment this  
B19 fragment corresponds to nucleotides 200 to 480 as  
numbered by Shade et al. (1986) and contains the entire  
5' non-coding region and p6 promoter of B19. This 280  
bp fragment is flanked by EcoRI and XbaI restriction  
10 sites and can be generated by cleavage with these  
restriction enzymes. This fragment is cloned into the  
EcoRI-XbaI sites of pUC19 to generate plasmid pB19p6.  
The skilled artisan will recognize that other plasmids  
and restriction sites can be utilized to generate a  
15 vector comprising a B19p6 promoter. Alternatively, the  
B19p6 promoter can be synthesized chemically or  
enzymatically based upon the published sequence and  
ligated to the heterologous gene.

A heterologous gene can be operably linked  
20 downstream of the B19p6 promoter fragment as follows.  
The plasmid pB19p6 is cleaved with HincII, which cleaves  
B19 DNA downstream of the p6 promoter (i.e. at  
nucleotide 424) and also in the multiple cloning site of  
pUC19. The desired heterologous gene is blunt-end  
25 ligated downstream from the B19p6 promoter between the  
two HincII sites to generate a plasmid pB19p6-insert.  
The ordinarily skilled artisan will recognize a variety  
of methods, as exemplified, e.g. in Sambrook et al.  
(1989), to ligate a fragment containing a cell-specific  
30 promoter with a fragment containing the heterologous  
gene. In accordance with the present invention, the  
coding sequence of GM-CSF, APRT, neo<sup>r</sup>, the

-19-

1    retinoblastoma gene,  $\alpha$ -globin,  $\beta$ -globin and  $\gamma$ -globin  
have been employed as the heterologous gene, resulting  
in the construction of hybrid vectors, designated AAV-  
B19-GM-CSF, AAV-B19-APRT, AAV-B19-neo $^r$ , AAV-B19-RB, AAV-  
5    B19- $\alpha$ -globin, AAV-B19- $\beta$ -globin and AAV-B-19-globin,  
respectively. A multidrug resistance gene encoding P-  
glycoprotein is also specifically contemplated as the  
heterologous gene. The coding sequences of the  
respective genes are known [Lee et al. (1985) Proc.  
10    Natl. Acad. Sci. USA 82, 4360 (GM-CSF); Broderick et al.  
(1987) Proc. Natl. Acad. Sci. USA 84, 3349 (APRT);  
Tratschin et al. (1985) Mol. Cell. Biol. 5, 3251 (Neo $^r$ );  
Huang et al. (1988) Science 242, 1563 (RB-1); Liebhaber  
et al. (1980) Proc. Natl. Acad. Sci. USA 77, 7054 ( $\alpha$ -  
15    globin); Lawn et al. (1980) Cell 21, 647 ( $\beta$ -globin);  
Enver et al. (1989) Proc. Natl. Acad. Sci. USA 86, 7033  
( $\gamma$ -globin) Roninson et al. (1986) Proc. Nat'l Acad. Sci.  
USA 83, 4538; Roninson et al. (1991) in Molecular and  
Cellular Biology of Multidrug Resistance in Tumor Cells  
20    (ed. Roninson, Plenum Press, NY) 91-106; Schinkel et al.  
(1991) Cancer Res. 51, 2628; Chen et al. (1990) J. Biol.  
Chem. 265, 506; (mdr)] and thus can be easily provided  
as described hereinabove.

The pB19p6-insert plasmid exemplifies a promoter-  
25    heterologous gene cassette which can be isolated by  
digesting the plasmid, for example, with EcoRI and  
HindIII or other appropriate restriction enzymes, and  
then ligated between two AAV-ITRs. The AAV-ITRs are  
provided by, for example, restriction digestion of AAV  
30    DNA or AAV cloned DNA, or chemical or enzymatic  
synthesis based upon the published sequence of AAV ITRs  
[Lusby et al. (1980)]. In a preferred embodiment, the

-20-

- 1 AAV ITRs comprise the 145 nucleotides shown in Fig. 1. Fragments which contain the 125 nucleotides which form the palindromic hairpin (nucleotide 1-125 of Fig. 1) or longer fragments which contain the terminal 191
- 5 nucleotides of the viral chromosome are also useful. Additional endogenous sequences, for example linkers to facilitate cloning and ligation, can also be used in the constructs. In a preferred embodiment, the AAV ITRs are provided by a plasmid, e.g. psub201 [Samulski et al.
- 10 (1987)] which is an AAV derivative into which XbaI cleavage sites have been introduced at sequence positions 190 and 4484, and the right-terminal 191 base pairs of the viral genome have been substituted for the normal left-terminal 190 base pair domain. This
- 15 modification results in the extension of the psub201 terminal repeats to 191 base pairs. The XbaI cleavage sites allow substitution of the AAV coding region with exogenous sequences, i.e. the B19 promoter and heterologous gene, such that the exogenous sequences are
- 20 flanked by the AAV-ITRs. Derivatives of psub201 engineered to contain other restriction sites, as demonstrated in Examples 2 and 3, are also useful for providing the AAV-ITRs.

To substitute the B19p6-insert, i.e. the

- 25 cassette, for the AAV coding region, psub201 is digested with XbaI to delete the AAV coding regions. Plasmid vector DNA containing the AAV-ITRs is isolated and ligated to the B19p6-insert construction. Ligation may be facilitated by the addition of adapters to the AAV-ITRs and linkers to the B19-p6-insert.

For example, in another preferred embodiment the vectors of the present invention are constructed by

-21-

1 modifying the engineered XbaI sites of psub201 to  
provide additional restriction sites, deleting the AAV  
coding regions by digestion with the appropriate  
restriction enzyme and ligating the B19p6-insert  
5 cassette to the plasmid DNA containing the AAV-ITRs.  
The construction of prototype plasmid vectors containing  
the AAV-ITRs but not the AAV coding regions, and which  
further contain cloning sites to facilitate the  
insertion of promoter-heterologous gene cassettes, are  
10 exemplified in Examples 2 and 3.

The resulting plasmid comprises a cell-specific  
promoter upstream of a heterologous sequence, both of  
which are flanked by AAV-ITRs. The order of the  
ligations, the nature of the complementary ends, the use  
15 of linkers and adapters, and other details can be varied  
as necessary by one of ordinary skill in the art to  
provide the AAV-B19 hybrid vector of the present  
invention.

To establish integration of the vector DNA into  
20 the chromosome of a host cell, host cells are  
transfected with the vector or infected with mature  
virions containing the hybrid vectors. Methods of DNA  
transfection are well-known to one of ordinary skill in  
the art and include, for example, naked DNA  
25 transfection, microinjection and cell fusion. More  
efficient integration is accomplished by infection with  
virions containing the hybrid vectors.

Virions can be produced by coinfection with a  
helper virus such as adenovirus, herpes virus or  
30 vaccinia virus. Following coinfection of host cells  
with the subject vector and a helper virus, virions are  
isolated and the helper virus is inactivated. The

-22-

1 resulting helper free stocks of virions are used to  
infect host cells. In another embodiment, virions are  
produced by cotransfected helper virus-infected cells  
with the vector of the present invention and a helper  
5 plasmid. For example, the hybrid construct of the  
present invention can be packaged into mature AAV  
virions by cotransfection of adenovirus-infected cells  
with the vector of the present invention and a plasmid  
which provides the parvovirus rep gene and adenovirus  
10 termini. An example of such a plasmid is pAAV/Ad, which  
contains the entire coding sequence of AAV and the  
adenovirus type 5 terminal sequences in place of the  
normal AAV termini. [Samulski et al. (1989)].  
Following cotransfection, mature virions are isolated by  
15 standard methods, e.g. cesium chloride centrifugation  
and heated at 56°C for our hour to inactivate any  
contaminating adenovirus. The resulting mature virions  
contain the vector of the present invention and are used  
to infect host cells in the absence of helper virus.  
20 Function of the hybrid vectors of the present  
invention, i.e. the ability to mediate transfer and  
expression of the heterologous gene in a specific cell  
type, can be evaluated by monitoring the expression of  
the heterologous gene in transduced cells. For example,  
25 bone marrow cells are isolated and enriched for  
hematopoietic stem cells (HSC), e.g. by fluorescence  
activated cell sorting as described in Srivastava et al.  
(1988) J. Virol. 62, 3059. HSC are capable of self-  
renewal as well as initiating long-term hematopoiesis  
30 and differentiation into multiple hematopoietic lineages  
in vitro. HSC are transfected with the vector of the  
present invention or infected with varying

-23-

1 concentrations of virions containing a subject hybrid vector and then assessed for the expression of the heterologous gene.

The assay for expression depends upon the nature  
5 of the heterologous gene. Expression can be monitored by a variety of methods including immunological, histochemical or activity assays. For example, Northern analysis can be used to assess transcription using appropriate DNA or RNA probes. If antibodies to the  
10 polypeptide encoded by the heterologous gene are available, Western blot analysis, immunohistochemistry or other immunological techniques can be used to assess the production of the polypeptide. Appropriate biochemical assays can also be used if the heterologous  
15 gene is an enzyme. For example, if the heterologous gene encodes antibiotic resistance, a determination of the resistance of infected cells to the antibiotic can be used to evaluate expression of the antibiotic resistance gene.

20 In addition to assessing that the heterologous gene is expressed in the appropriate cells, the correct promoter specificity of the hybrid vectors can be evaluated by monitoring the expression of the heterologous gene, or lack of expression, in cells in  
25 which the promoter is not expected to be active. For example, when cells from a naso-pharyngeal cell line, KB, are transduced with a hybrid vector containing the B19p6 promoter, the heterologous gene is not expressed, since the B19p6 promoter is erythroid cell-specific.  
30 Detection of the heterologous gene product at levels at or below the level of untransduced cells confirms that the B19p6 promoter of the hybrid vector does not direct

-24-

1 expression of the heterologous gene in non-hematopoietic  
cells.

The hybrid vectors of the present invention are useful for gene therapy. In particular, the vectors of  
5 the present invention can direct erythroid cell-specific expression of a desired gene, and thus are useful in the treatment of hemoglobinopathies.

It is contemplated in accordance with the present invention to use the hybrid vector in the treatment of a variety of diseases, including thalassemia, sickle-cell anemia, diabetes and cancer. The heterologous gene can be the normal counterpart of one that is abnormally produced or underproduced in the disease state, for example  $\beta$ -globin for the treatment of sickle-cell  
10 anemia, and  $\alpha$ -globin,  $\beta$ -globin or  $\gamma$ -globin in the treatment of thalassemia. The heterologous gene can encode antisense RNA as described hereinabove. For example,  $\alpha$ -globin is produced in excess over  $\beta$ -globin in  $\beta$ -thalassemia. Accordingly,  $\beta$ -thalassemia can be  
15 treated in accordance with the present invention by gene therapy with a vector in which the heterologous gene encodes an antisense RNA. The antisense RNA is selected such that it binds to a target sequence of the  $\alpha$ -globin mRNA to prevent translation of  $\alpha$ -globin, or to a target  
20 sequence of the  $\alpha$ -globin DNA such that binding prevents transcription of  $\alpha$ -globin DNA. In the treatment of cancer the heterologous gene can be a gene associated with tumor suppression, such as retinoblastoma gene, the anti-oncogene p53, or the gene encoding tumor necrosis  
25 factor.  
30

The use of the hybrid vectors of the present invention for the treatment of disease involves

-25-

1 transduction of HSC or progenitor cells with the hybrid  
vector. Transduction is accomplished by transfection  
with the vector or preparation of mature virions  
containing the hybrid vectors and infection of HSC or  
5 progenitor cells with the mature virions. Transduced  
cells are introduced into patients, e.g. by intravenous  
transfusion (see, for example, Rosenberg, 1990). HSC or  
progenitor cells are provided by obtaining bone marrow  
cells from patients and optionally enriching the bone  
10 marrow cell population for HSC. HSC can be transduced  
by standard methods of transfection or infected with  
mature virions for about one to two hours at about 37°C.  
Stable integration of the viral genome is accomplished  
by incubation of HSC at about 37°C for about one week to  
15 about one month. The stable, site-specific integration  
and erythroid cell-specific expression is assessed as  
described above. After the transduced cells have been  
introduced into a patient, the presence of the  
heterologous gene product can be monitored or assessed  
20 by an appropriate assay for the gene product in the  
patient, for example in peripheral red blood cells or  
bone marrow of the patient when expression is erythroid  
cell-specific. As described hereinabove, the specific  
assay is dependent upon the nature of the heterologous  
25 gene product and can readily be determined by one  
skilled in the art.

For example, β-thalassemia represents a  
heterologous group of clinical syndromes that are  
inherited as mutated alleles of genes that encode the  
30 human β-globin chain. These mutations affect all  
aspects of β-globin gene expression including  
transcription, splicing, polyadenylation, translation

-26-

1 and protein stability. The hallmark of  $\beta$ -thalassemia is  
the marked reduction or total absence of synthesis of  
normal adult hemoglobin (HbA;  $\alpha_2\beta_2$ ). Despite  
significant advances in the understanding of basic  
5 underlying molecular mechanisms of  $\beta$ -thalassemia,  
treatment is limited to regular red blood cell  
transfusions and iron-chelation therapy. Treatment by  
bone marrow transplantation has also been attempted  
[Thomas *et al.* (1982) *Lancet*, *ii*, 227], but an effective  
10 cure has not been found.

Accordingly, the vectors of the present invention  
are useful in the treatment of  $\beta$ -thalassemia. An AAV-  
B19 vector is constructed in which the heterologous gene  
is the normal human  $\beta$ -globin gene, with the resulting  
15 AAV-B19- $\beta$ -globin vector allowing parvovirus-mediated  
transfer, site-specific integration and erythroid cell-  
specific expression of the normal human  $\beta$ -globin gene in  
human hematopoietic cells.

Abnormal  $\beta$ -globin expression  $\beta$ -thalassemia may  
20 result in the overabundance of  $\alpha$ -globin mRNA relative to  
 $\beta$ -globin mRNA. The present invention cannot only  
provide a normal  $\beta$ -globin gene, as described  
hereinabove, but can further be utilized to down-  
regulate the production of excess  $\alpha$ -globin by providing  
25 a vector with an antisense RNA as the heterologous gene.  
An AAV-B19 hybrid vector is constructed in which the  
heterologous sequence encodes an antisense RNA which is  
sufficiently complementary to a region of the mRNA  
encoding the  $\alpha$ -chain, such that it binds to and prevents  
30 translation of the  $\alpha$ -globin mRNA, or to a region of the  
DNA encoding  $\alpha$ -globin such that it binds to and prevents  
transcription of the  $\alpha$ -globin gene. Hence, the present

-27-

1 invention contemplates gene therapy for  $\beta$ -thalassemia comprising transduction of hematopoietic stem or progenitor cells with a hybrid vector encoding normal  $\beta$ -globin chains, or simultaneous transduction with a  
5 vector encoding a normal  $\beta$ -globin chain and a vector encoding an RNA antisense to  $\alpha$ -globin mRNA or DNA. Alternately, a construction with more than one B19p6 promoter, as described hereinabove, permits coincident expression of  $\beta$ -globin and antisense  $\alpha$ -globin.  
10 Accordingly, transduction with a single vector effects both the provision of a normal  $\beta$ -globin gene and the down-regulation of excess  $\alpha$ -chains. More specifically, bone marrow cells are transfected with the subject vectors, and transduced cells are introduced, e.g. by  
15 intravenous transfusion, into a patient. The stable integration of the vector can be assessed by PCR or Southern blot analysis and the expression of the heterologous gene can be evaluated by assaying for the heterologous gene product in the patient's peripheral  
20 blood cells or bone marrow cells. As described previously, the particular assay depends upon the nature of the heterologous gene product.

The vectors of the present invention are also useful in conferring cell specific multidrug resistance.  
25 An AAV vector is constructed to contain a cell-specific promoter and a multidrug resistance gene, with the resulting vector allowing parvovirus-mediated transfer, site-specific integration and cell-specific expression of the mdr gene in a selected cell type. In a preferred  
30 embodiment, the vector is AAV-B19-mdr, and confers the multidrug resistance phenotype to erythroid cells.

-28-

1 Any of the numerous mdr genes known to confer the  
mdr phenotype is useful as the heterologous gene. The  
mdr genes are known to the ordinarily skilled artisan  
and are described, for example, by Roninson *et al.*  
5 (1991) in Molecular and Cellular Biology of Multidrug  
Resistance in Tumor Cells (ed. Roninson, Plenum Press,  
NY) 91-106. Accordingly, the present invention provides  
a method of conferring cell-specific multidrug  
resistance which comprises transducing cells with the  
10 hybrid vector of the present invention which contains a  
cell-specific promoter and an mdr gene. In a preferred  
embodiment, the present invention provides a method of  
conferring multidrug resistance to erythroid cells of a  
patient which comprises obtaining bone marrow or stem  
15 cells from a patient, transducing the bone marrow or  
stem cells with the AAV-B19 hybrid vector of the present  
invention which contains an mdr gene as the heterologous  
gene, and reintroducing the transduced cells into a  
patient. The expression of the mdr gene can be  
20 evaluated by assaying for the mdr gene product, i.e.,  
P-glycoprotein, in the patient's peripheral blood cells  
or bone marrow cells. For example, P-glycoprotein can  
be detected by known immunologic assays with an antibody  
against P-glycoprotein. Such antibodies are known and  
25 available to the ordinarily skilled artisan, and are  
described, for example, by Meyers *et al.* (1989) Cancer  
Res. 49, 3209 and Georges *et al.* (1990) Proc. Nat'l  
Acad. Sci. USA 87, 152. The present method is  
particularly useful as an adjunct to cancer chemotherapy  
30 in that it effectively results in the protection of  
erythroid cells from the effects of chemotherapeutic  
agents targeted to other tissues.

-29-

1        Yet another aspect of the present invention  
provides a method for delivery of a pharmaceutical  
product, a protein or an antisense RNA in a mammal.  
Since the normal differentiation of these stem cells  
5      results in production of mature erythrocytes, the  
transduction of stem cells with the subject vector  
ultimately yields a population of circulating, enucleate  
vesicles containing the gene product. This method  
comprises transducing hematopoietic stem or progenitor  
10     cells with the hybrid vector of the present invention  
and introducing, e.g. by intravenous transfusion or  
injection, the transduced cells into a mammal.  
Transduction can be accomplished by transfecting cells  
with the hybrid vector by standard methods of infecting  
15     cells with mature AAV virions containing the hybrid  
vector at about 37°C for about one to two hours. Stable  
integration of the recombinant viral genome is  
accomplished by incubating cells at about 37°C for about  
one week to about one month. Transduced cells are  
20     recognized by assaying for expression of the  
heterologous gene, as described hereinabove. In this  
embodiment, the pharmaceutical product is encoded by the  
heterologous gene of the hybrid vector, and can be any  
pharmaceutical product capable of being expressed by the  
25     hybrid vector. Such products include α, β and γ-globin,  
insulin, GM-CSF, M-CSF, G-CSF, EPO, TNF, MGF,  
interleukins, the gene product of the retinoblastoma  
gene, p53 or adenosine deaminase. Therefore, the  
present invention can provide production of constitutive  
30     levels of heterologous gene products inside membrane  
vesicles, specifically red blood cells, for in situ  
treatment of disease. Optionally, the hybrid vector can

-30-

- 1 further comprise a sequence which encodes a signal peptide or other moiety which facilitates the secretion of the gene product from the erythroid cell. Such sequences are well-known to one of ordinary skill in the art [see, for example, Michaelis *et al.* (1982) Ann. Rev. Microbiol. 36, 435] and can be inserted into the subject vectors between the promoter and coding region by methods described herein. This method can be used to treat a variety of diseases and disorders and is not limited to the treatment of hemoglobinopathies, since the heterologous gene is constitutively expressed and can be released from the red blood cell by virtue of a secretory sequence, or released when red blood cells are lysed in the liver and spleen.
- 15 The following examples further illustrate the present invention.

20

25

30

35

-31-

1

EXAMPLE 1

Site-Specific Integration  
of the Recombinant AAV Genome

5 Site-specific integration of the AAV genome was confirmed by an approach in which normal human diploid fibroblasts (HDF) were either mock-infected, or infected with an increasing multiplicity-of-infection (moi) of wild-type AAV. Following multiple serial passage of  
10 these cells in culture, their total genomic DNA was isolated, digested with a variety of restriction endonucleases, and analyzed on Southern blots using an AAV-specific DNA probe. A representative Southern blot is presented in Fig. 4. Restriction enzymes are  
15 indicated at the top of the figure. The moi is indicated at the top of each lane, with 0.0 indicating mock-infection. The predominant single band of hybridization is evidence that the wild-type AAV genome integrates into normal human diploid cell chromosomal  
20 DNA in a site-specific manner. The target site was saturated only at very high moi of AAV, and no selection procedure was employed to select for cell populations that have the integrated provirus.

25 The site-specific integration of the recombinant AAV genome is demonstrated utilizing human bone marrow cells, which are the target cells for therapy of hemoglobinopathies.

30 Bone marrow cells were obtained from hematologically normal volunteer donors, and low-density, mononuclear bone marrow (LDBM) cells were isolated by Ficoll-Hypaque density centrifugation. LDBM cells were infected with the recombinant AAV-Neo virions

-32-

1 (vSV40-Neo), in which the Neo gene, under th control of  
the SV40 early promoter, is encapsidated into AAV  
particles, and incubated in the presence of various  
cytokines such as GM-CSF (1 ng/ml) and IL-3 (1 ng/ml)  
5 for 48 hours. The cells were incubated in liquid  
cultures in the presence of G418 at 37°C for 10 days,  
their total genomic DNA was isolated, cleaved with  
BamHI, and analyzed on a Southern blot using a Neo-  
specific DNA probe as shown in Fig. 5. Concentration of  
10 G418 is indicated at the top of each lane. The single  
band of hybridization indicated by the arrow  
demonstrates that the recombinant AAV viral genome  
undergoes site-specific integration into human bone  
marrow cell chromosomal DNA.

15

20

25

30

35

-33-

1

EXAMPLE 2Construction of Recombinant  
Plasmids pWP-7A and pWP-19

5       The general overall strategy used to construct the prototype plasmid vectors, designated pWP-7A and pWP-19, respectively, is depicted in Figure 6. The XbaI sites in plasmid psub201 were converted to EcoR1 sites by ligating synthetic XbaI-EcoR1-XbaI adaptors as described by Srivastava *et al.* (1989) Proc. Natl. Acad. Sci. USA 86, 8078. The AAV coding region was removed following digestion with EcoR1, and the vector DNA containing the two AAV-ITRs was isolated from preparative agarose gels (Seth [1984] Gene Anal. Tech. 1, 99), and treated with PolIk to generate blunt-ends. Similarly, pBR322 DNA was cleaved with EcoR1 and PvuII and a 2066 bp fragment containing the entire coding region of a gene for resistance to tetracycline ( $Tc^R$ ) was also blunt ended with PolIk. These two fragments were ligated and used to transform competent E. coli HB101 cells by the standard methods described in Sambrook *et al.* (1989) to generate a plasmid, designated pWP-7A. Since blunt-end ligation of DNA fragments containing repaired EcoR1 and PvuII ends regenerates an EcoR1 site, plasmid pWP-7A can be cleaved with EcoR1 downstream from the  $Tc^R$  gene for cloning a gene or cassette of interest. The  $neo^R$  gene under the control of the herpesvirus thymidine kinase (TK) promoter was isolated from plasmid pSHL-172 (Tratschin *et al.* [1985] Mol. Cell. Biol. 5, 3251) by partial digestion with PvuII, and blunt-end ligated with PolIk-treated pWP-7A DNA. The resulting recombinant plasmid, designated pWP-

-34-

1 8A, is shown in Figure 6-A. Plasmid pWP-19 was  
constructed as follows. Plasmid pWP-8A was linearized  
with HindIII, which cleaves at the 5' end of the Tc<sup>R</sup>  
gene, and partially digested with XmnI to remove the Tc<sup>R</sup>  
5 gene. A plasmid pGBOR which contains a gene for  
resistance to ampicillin (Ap<sup>R</sup>) and the bacteriophage  
lambda operator (OR1/OR2;λo) sequences (Samulski *et al.*  
[1991] EMBO J. **10**, 3941) was cleaved with EcoRI and XbaI  
and the fragment containing the λo sequence was blunt-  
10 end ligated with P<sub>O</sub>LIk-treated pWP-8A DNA described  
above. The resulting recombinant plasmid pWP-19 is  
shown in Figure 6-B.

The plasmid vectors pWP-7A and pWP-19 are useful  
for constructing recombinant AAV genomes because direct  
15 insertion of an insert of interest is possible in both,  
and the presence of the built-in neo<sup>R</sup> gene in pWP-19  
provides a strong selectable marker in human cells. The  
plasmid vector pWN-1 is particularly useful because it  
offers several features. In bacterial cells, these  
20 include: 1) The availability of a variety of cloning  
sites (EcoRI, BamHI, SacI, KpnI, XbaI, SalI, AccI,  
HincII, PstI, SphI and HindIII), including the NdeI  
site; 2) AAV-ITRs which are well separated from the  
cloning sites; and 3) the use of Tc<sup>R</sup> as well as Ap<sup>R</sup> as  
25 selectable markers. In mammalian cells, following  
transfection in the presence of the AAV helper plasmid  
and Ad, the insert of interest, which is now flanked by  
the two AAV-ITRs in their proper orientation (see Figure  
7), can be efficiently rescued from the Tc<sup>R</sup> gene  
30 followed by DNA replication and packaging in the AAV  
progeny virions.

-35-

1        Whereas plasmid pWP-7A is useful for cloning  
inserts up to 2.5 kb in size at the unique EcoR1 site,  
plasmid pWP-19 offers a built-in neo<sup>R</sup> marker gene as  
well as a number of cloning sites such as BamH1, SacI  
5        and KpnI, and insert up to 2.6 kb in size can be  
inserted between the two AAV-ITRs.

10

15

20

25

30

35

-36-

1

EXAMPLE 3

Construction of Recombinant Plasmid pWN-1

The strategy to construct the recombinant plasmid  
5 pWN-1 is shown in Figure 7. Briefly, psub201 plasmid  
DNA was digested to completion with XbaI and PvuII, and  
a 191-bp XbaI-PvuII fragment containing the entire AAV-  
ITR sequence was isolated as described in Example 2.  
Similarly, pBR322 plasmid DNA was cleaved with EcoR1 and  
10 AvaI to isolate a 1425-bp fragment that contains the Tc<sup>R</sup>  
gene but lacks the origin of DNA replication ("ori")  
sequence. This fragment was treated with PolIk to  
generate blunt ends. Blunt-ended EcoR1-AvaI fragment  
was mixed with a large excess of the XbaI-PvuII fragment  
15 containing the AAV-ITR, blunt-end ligated using T4 DNA  
ligase, and then digested exhaustively with XbaI. This  
resulted in the production of the Tc<sup>R</sup> gene flanked by a  
single AAV-ITR at each end but in the opposite  
orientation (see small arrows in boxed ITRs). This  
20 fragment was subsequently ligated at the unique XbaI  
site in plasmid pGBOR described above, and Tc<sup>R</sup> was used  
to select for the recombinant plasmid pWN-1.

25

30

35

**SUBSTITUTE SHEET**

-37-

1

EXAMPLE 4Rescue and Replication of a Cloned  
Insert from Recombinant AAV-Based Plasmids

5 DNA sequences flanked by the two AAV-ITRs can be rescued from the recombinant plasmids of Examples 2 and 3 following transfection in human cells in the presence of the AAV and Ad proteins as a prelude to successful packaging of these genes into mature AAV virions. The  
10 insert size between the two AAV-ITRs in plasmid pWP-8A is similar to that of the wt AAV genome. The AAV-rep gene the parent plasmid psub201, as well as the λ OR1/OR2 sequences from plasmid pGBOR were isolated and inserted in plasmid pWP-19 by the strategy shown in  
15 Figure 8-A. Two recombinant plasmids, pWP-21 and pWP-22, were generated which contain the AAV-rep gene in different orientations with respect to the neo<sup>R</sup> gene. These plasmids are depicted in Figure 8-A. Similarly,  
20 the insert size between the two AAV-ITRs in plasmid pWN-1 was increased by inserting the neo<sup>R</sup> gene either at the NdeI site or at the PstI site to generate two recombinant plasmids, pWP-16 and pWP-17, respectively, which are shown in Figure 8-B.

Plasmids pWP-8A, pWP-21, pWP-22, pWP-16 and pWP-25 17 were either transfected alone, or co-transfected with the AAV helper plasmid (pAAV/Ad) separately, in Ad-infected human KB cells (Samulski *et al.* [1989] *J. Virol.* **63**, 3822; Srivastava *et al.* [1989]; Srivastava [1990] *Blood* **76**, 1997). Low M<sub>r</sub> DNA samples isolated by  
30 the method described by Hirt (1967) *J. Mol. Biol.* **26**, 365, were digested with DpnI and analyzed on Southern blots (Southern [1975] *J. Mol. Biol.* **98**, 503) using a

-38-

1 neo-specific DNA probe as previously described (Samulski  
et al. 1989). The results are presented in Figure 9.  
No rescue/replication of the recombinant neo<sup>R</sup> gene from  
plasmid pWP-8A occurred in the absence of the pAAV/Ad  
5 helper plasmid (Lane 1); successful rescue and  
replication indeed occurred when the AAV-Rep proteins  
were supplied in trans (Lane 2), as detected by the  
presence of the characteristic monomeric and dimeric  
replicative intermediates of the recombinant AAV genome.  
10 Similarly, rescue and replication occurred from plasmids  
pWP-21 (Lanes 3 and 4), and pWP-22 (Lanes 5 and 6) even  
in the absence of the helper plasmid because these  
plasmids contain the AAV-rep gene in cis. Rescue and  
replication from plasmids pWP-16 and pWP-17 also  
15 occurred, but only in the presence of the AAV helper  
plasmid (Lanes 8 and 10).

Following rescue and replication, the neo<sup>R</sup> gene  
could also be packaged into mature AAV progeny virions  
in presence of the AAV-Cap proteins. The recombinant  
20 AAV progeny virions were biologically active and  
infectious. For example, recombinant AAV-neo virions  
were used to transduce and stably integrate the neo<sup>R</sup>  
gene in a variety of diploid and polyploid human cells.  
The transduced neo<sup>R</sup> gene was biologically active, as  
25 determined by gene expression analyses on Northern  
blots, as well as by ready isolation of clonal  
populations of human cells that were resistant to  
geneticin.

30

35

-39-

1

EXAMPLE 5

Construction of an AAV-B19 Hybrid  
Parvovirus Cloning and Expression Vector

5       A hybrid vector containing the AAV-ITRs the B19p6 promoter and the neo<sup>r</sup> gene as the heterologous gene was constructed as follows. The general strategy for the construction of this vector, designated pB19-p6-Neo<sup>r</sup>-AAV-ITR, is shown in Fig. 10.

10       A near full-length B19 DNA clone (for example pYT104v in which B19 is downstream of the bacteriophage SP6 promoter) was cleaved with SacI and DraI, and the small DNA fragment that contains the left ITR was discarded. Following blunt-end re-ligation of the 15 larger fragment, the plasmid DNA was cleaved with EcoRI and XbaI to isolate the 280 bp fragment that contains the entire 5' non-coding region and the p6 promoter of B19. This fragment was cloned into the EcoRI-XbaI sites of pUC19 to generate a plasmid pB19p6. This plasmid was 20 cleaved with HincII which digests the B19 DNA downstream from the p6 promoter and also the pUC19 DNA in the multiple cloning site. The bacterial Neo<sup>r</sup> gene (Tratschin *et al.*, 1985) was blunt-end ligated downstream from the B19p6 promoter between the two 25 HincII sites to generate the plasmid pB19p6-Neo<sup>r</sup>. This plasmid was digested with EcoRI and HindIII and the B19p6-Neo insert was isolated and ligated between the two AAV-ITRs of XbaI digested psub201. The vector was 30 packaged into mature AAV virions (vB19-Neo) by cotransfection of adenovirus infected cells with pAAV/Ad, which contains the AAV-coding sequence and the

-40-

1 adenovirus type 5 terminal sequences (Samulski t al.  
1989).

5

10

15

20

25

30

35

**SUBSTITUTE SHEET**

-41-

1

EXAMPLE 6

Recombinant Parvovirus-Mediated Transfer of  
Bacterial Neo<sup>r</sup> Gene in Human Hematopoietic Stem Cells

5       Human hematopoietic stem cells were isolated from normal volunteer donors followed by sorting with monoclonal antibodies against the human CD34 and DR antigens according to the method of Lu et al. (1987) J. Immunol. 139, 1823 to produce a CD34<sup>+</sup>DR<sup>-</sup> cell population. This cell population is known to contain several classes of primitive human hematopoietic progenitor cells including colony forming unit-blast cells (CFU-B1), high-proliferative potential colony forming cells (HPP-CFC), and cells responsible for initiating long-term hematopoiesis in vitro (LTBMIC).

10      Approximately  $1 \times 10^3$  CD34<sup>+</sup>DR<sup>-</sup> cells isolated from two different donors were either mock-infected, or infected at varying moi with vTK-Neo or vB19-Neo virions. (vTK-Neo is recombinant AAV virion containing the Neo<sup>r</sup> gene under the control of the thymidine kinase (TK) promoter). Cells were incubated at 37°C for one week in the presence of the cytokines interleukin-3 (1 ng/ml), granulocyte macrophage colony stimulating factor (1 ng/ml), and a factor for c-kit ligand termed mast cell growth factor (50 ng/ml). G418 was added at a final concentration of 250 µg/ml. The total number of viable cells was counted following one-week exposure to the drug. The concentration of G418 was then increased to 500 µg/ml, and viable cell counts were obtained after two weeks for vTK-Neo-infected cells, and after one week for vB19-Neo-infected cells. These data are shown in Fig. 11.

35

**SUBSTITUTE SHEET**

-42-

1       Exposure to vTK-Neo virions resulted in a nearly  
10-fold increase in the G418-resistant hematopoietic  
cell population compared with mock-infected cells,  
whereas the exposure to the vB19-Neo virions resulted in  
5      approximately 4-fold increase at the highest moi of the  
virions compared with mock infected cells. These  
results demonstrate that the B19p6 promoter is active in  
cell populations enriched for HSC, albeit at a lower  
level compared with the TK promoter.

10

15

20

25

30

35

-43-

1

EXAMPLE 7Evaluation of Tissue-Specificity of the B19p6 Promoter

Non-erythroid cells were infected to determine whether the B19p6 promoter in the hybrid constructions had become indiscriminate or had maintained its erythroid specificity.

Human KB cells were either mock-infected or infected separately with equivalent moi of vTK-Neo and 10vB19-Neo. At 48 hours post-infection cells were exposed to various concentration of G418. Following a 14-day incubation period at 37°C, the approximate numbers of G418-resistant colonies were enumerated. A colony is defined as a group of eight or more cells. These data are presented in Table 1, and demonstrate that under conditions of viral infection, the B19p6 promoter retains its erythroid-specificity.

TABLE 1

Approximate Numbers of G418-Resistant Colonies in KB Cells Transduced with AAV-Neo Virions

Recombinant virus	200 µg/ml G418	400 µg/ml G418	600 µg/ml G418
1. None	10-20	0	0
2. vTK-Neo	TMTC*	100-200	50-100
3. vB19-Neo	10-20	0	0

\*TMTC = Too many to count

30

35

-44-

1

EXAMPLE 8

Construction of Parvovirus Vectors  
with an Erythroid-Specific Enhancer

5        In order to further increase the tissue-specific expression directed by the B19p6 promoter, the DNasel-Hypersensitive Site-2 (HS-2) of the Locus Control Region (LCR), (Tuan et al. (1985) Proc. Natl. Acad. Sci. USA 82, 6384), an erythroid-specific enhancer, was inserted  
10      into the hybrid vectors of the present invention. As diagrammed in Fig. 12, the HS-2 gene is inserted upstream of the B19p6 promoter and luciferase gene to provide the vector vHS2/B19-Luc. Restriction sites used to facilitate vector construction are shown in Fig. 12.  
15      These constructs were packaged into mature AAV virions.

20

25

30

35

-45-

1

EXAMPLE 9

Construction of Parvovirus Vectors  
Containing the Normal Human  $\beta$ -Globin Gene

5        In order to provide vectors for gene transfer in clinical cases of  $\beta$ -thalassemia and sickle-cell anemia, two plasmid vectors that contain the normal human  $\beta$ -globin gene were constructed and packaged into recombinant AAV virions. The pHS2/ $\beta$ -globin-Neo

10      construct contains the  $\beta$ -globin promoter and the upstream HS-2 enhancer, along with the Neo<sup>r</sup> gene under the control of the TK promoter, and the pHS2/B19-globin-Neo construct also contains the B19p6 promoter. These constructs are shown in Fig. 13.

15      The pHS2/ $\beta$ -globin-Neo vector was constructed as follows. A plasmid (pWP19) was constructed that contains the Neo<sup>r</sup> gene under the TK promoter between the two AAV-ITRs. pWP19 was linearized with SacI. A SnaBI-PstI fragment containing the genomic clone of the human 20  $\beta$ -globin gene was ligated, in reverse orientation, upstream of the TK promoter. The resulting plasmid was linearized by digestion with KpnI, and a HindIII-XbaI fragment containing the HS2 enhancer was ligated upstream of the  $\beta$ -globin promoter.

25      The pHS2/B-19-globin-Neo vector was constructed as follows. First the HindIII-XbaI fragment containing the HS2 enhancer was cloned upstream of the B19p6 promoter in the plasmid pB19p6 by linearizing it with EcoRI. The HS2-B19p6 fragment was isolated by digesting 30 this plasmid with PvuII and HincII. The PvuII-HincII fragment was ligated to pWP19 plasmid linearized with SacI. Second, the  $\beta$ -globin coding region lacking the  $\beta$ -

-46-

1 globin promoter was excised by digesting the plasmid  
with NcoI and PstI. This NcoI-PstI fragment was ligated  
to the plasmid described above by linearizing it with  
KpnI.

5 Similarly, vectors containing the human  $\alpha$ -globin  
gene in both orientations were constructed and packaged  
into recombinant AAV virions. A HinFI-PvuII fragment of  
the cloned  $\alpha$ -globin gene (Liebhaber, 1980) was ligated  
downstream from the B19p6 promoter following

10 linearization of the pB19p6 plasmid DNA. The resulting  
plasmid was linearized with FspI, and ligated with the  
pWP19 plasmid at the SacI site prior to packaging into  
the AAV virions.

15

20

25

30

35

-47-

1

EXAMPLE 10

AAV Tranduction of Human Cells  
with the Multidrug Resistance Gene

5        To assess the ability of AAV vectors to confer the multidrug resistance phenotype to drug sensitive human cells, drug sensitive human KB cells are transduced with the AAV vector containing an mdr gene under the control of the thymidine kinase promoter. The 10 expression of the mdr phenotype is assessed by detection of P-glycoprotein with an antibody specific for this protein. Further, the ability of the transduced cells to reduce intracellular accumulation of certain anti-tumor drugs is evaluated by culturing transduced cells 15 in a series of tissue culture media containing step-wise concentration increases of the selected drug. An increase in viability of transduced cells relative to control (untransduced) cells is indicative of expression of the mdr gene.

20       To evaluate the ability of the AAV vectors of the present invention to confer cell-specific multidrug resistance, both erythroid and non-erythroid (e.g. drug sensitive KB) cells are transduced with the AAV vector containing an mdr gene under the control of the B19p6 promoter (AAV-B19p6-mdr). KB cells are transduced and 25 assessed as described above. Since the B19p6 promoter directs erythroid cell-specific expression, the mdr gene is not expressed by transduction of KB cells with AAV-B19p6-mdr.

30       The ability of AAV-B19p6-mdr to confer erythroid cell-specific multidrug resistance is assessed as follows. Bone marrow is removed from a patient,

35

-48-

- 1 enriched to CD34<sup>+</sup> cells and transduced with AAV-B19p6-mdr. Primary cultures are then established and maintained until focal colonies can be transferred to tissue culture trays. Outgrowth of such colonies
- 5 permits the assessment of resistance to a range of anti-tumor drugs.

The AAV-B19p6-mdr vector is useful in cancer chemotherapy. For example, bone marrow is removed from the iliac creast of a cancer patient, and enriched for CD34<sup>+</sup> cells. Enriched cells are transduced by AAV-B19p6-mdr and reinfused into the patient.

The B19p6 promoter can be substituted by other cell-specific promoter elements to permit expression of the multidrug resistance phenotype in other cell lineages.

20

25

30

35

-49-

1

EXAMPLE 11

Cells of a human erythroleukemia cell line, K562 (ATCC CCL-243) were infected with vHS2/B19-globin-Neo 5 (see Example 9 and Fig. 13) to assess the ability of the vector to provide expression of the  $\beta$ -globin gene in a host cell which exhibits no  $\beta$ -globin gene expression.

K562 cells were either mock-infected or infected separately with equivalent moi of vHS2/B19-globin-Neo 10 and vHS2/ $\beta$ -globin-Neo. DNA of infected cells was isolated, digested with NcoI, and subjected to Southern blot analysis. Fig. 14 provides a Southern blot in which Lane 1 corresponds to DNA isolated from mock-infected cells, and Lane 2 corresponds to DNA isolated 15 from cells infected with vHS2/ $\beta$ -globin-Neo. The blots were probed with a  $\gamma$ -globin probe (left panel) and a  $\beta$ -globin probe (right panel). The arrow denotes the transduced allele of the  $\beta$ -globin gene in K562 cells infected with vHS2/ $\beta$ -globin-Neo. Similar results were 20 obtained for K562 cells infected with vHS2/B19-globin-Neo.

The  $\beta$ -globin gene is present but not expressed in K562 cells. Northern blots of total RNA from the mock-infected and infected cells described above were probed 25 with a neomycin probe (Fig. 15, left panel) and a  $\beta$ -globin probe (Fig. 15, right panel). The center panel of Fig. 15 presents an ethidium-bromide stained gel. Lane 1 in each panel represents RNA from mock-infected cells; Lanes 2 and 3 correspond to RNA from vHS2/ $\beta$ -globin-Neo infected cells; Lanes 4 and 5 correspond to 30 RNA from vHS2/B19-globin-Neo infected cells. Plus and minus signs indicate the presence or absence,

-50-

1 respectively, of hemin, an inducer of  $\gamma$ -globin which  
appeared to have no effect on  $\beta$ -globin expression. The  
Northern analysis indicates that the  $\beta$ -globin gene is  
expressed in K562 cells infected with vHS2/ $\beta$ -globin-Neo  
5 and vHS2/B19-globin-Neo, but not in mock-infected cells.

10

15

20

25

30

35

**SUBSTITUTE SHEET**

-51-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Srivastava, Arun

(ii) TITLE OF INVENTION: SAFE VECTOR FOR GENE THERAPY

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Scully, Scott, Murphy Presser

(B) STREET: 400 Garden City Plaza

(C) CITY: Garden City

(D) STATE: New York

(E) COUNTRY: USA

(F) ZIP: 11530

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release q.0, Version q.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: McNulty, William E.

(B) REGISTRATION NUMBER: 22,606

(C) REFERENCE/DOCKET NUMBER: 8361

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (516) 742-4343

(B) TELEFAX: (516) 742-4366

(C) TELEX: 230 901 SANS UR

-52-

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 145 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTGGCCACTC CCTCTCTGCG CGCTCGCTCG CTCACTGAGG CCGGGCGACC AAAGGTCGCC	60
CGACGCCCGG GCTTGGCCCG GGCGGCCTCA GTGAGCGAGC GAGCGCGCAG AGAGGGAGTG	120
GCCAACCTCCA TCACTAGGGG TTCCCT	145

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 225 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTTAGCGGG CTTTTTCCC GCCTTATGCA AATGGGCAGC CATTAAAGT GTTTTACTAT	60
AATTTTATTG GTTAGTTTG TAACGGTTAA AATGGGCGGA GCGTAGGCGG GGACTACAGT	120
ATATATAGCA CGGTACTGCC GCAGCTCTT CTTTCTGGGC TGCTTTTCC TGGACTTTCT	180
TGCTGTTTTT TGTGAGCTAA CTAACAGGTA TTTATACTAC TTGTT	225

1    WHAT IS CLAIMED:

1. An expression vector for site-specific integration and cell-specific gene expression comprising two inverted terminal repeats of adeno-associated virus 2 and at least one cassette comprising a promoter capable of effecting cell-specific expression wherein said promoter is operably linked to a heterologous gene, and wherein said cassette resides between said inverted terminal repeats.
- 5    2. The vector of Claim 1 wherein each of said inverted terminal repeats comprises the nucleotides of SEQ ID NO:1.
- 10    3. The vector of Claim 1 wherein each of said inverted terminal repeats comprises nucleotides 1 to 125 of SEQ ID NO:1.
- 15    4. The vector of Claim 1 wherein said promoter is a B19 parvovirus promoter.
- 20    5. The vector of Claim 4 wherein said B19 parvovirus promoter is the p6 promoter.
- 25    6. The vector of Claim 4 wherein said B19 parvovirus promoter comprises the nucleotides of SEQ ID NO:2.
- 30    7. The vector of Claim 1 wherein said heterologous gene encodes a biologically functional protein.
- 35    8. The vector of Claim 1 wherein said heterologous gene encodes a non-biologically functional protein.
- 40    9. The vector of Claim 1 wherein said heterologous gene encodes an antisense RNA.

1           10. The vector of Claim 1 wherein said  
heterologous gene is selected from the group consisting  
of a gene encoding  $\alpha$ -globin,  $\beta$ -globin,  $\gamma$ -globin,  
granulocyte macrophage-colony stimulating factor (GM-  
5 CSF), tumor necrosis factor (TNF), any one of  
interleukins 1-11, neomycin resistance, luciferase,  
adenine phosphoribosyl transferase (APRT),  
retinoblastoma, insulin, mast cell growth factor, p53,  
adenosine deaminase.

10          11. The vector of Claim 1 wherein said  
heterologous gene encodes P-glycoprotein.

12. The vector of Claim 9 wherein said antisense  
RNA is complementary to a segment of the DNA or RNA  
encoding  $\alpha$ -globin.

15          13. The vector of Claim 5 wherein said vector is  
AAV-B19-GM-CSF, AAV-B19-APRT, AAV-B19-neo<sup>r</sup>, AAV-B19-RB,  
AAV-B19- $\beta$ -globin, AAV-B19- $\alpha$ -globin, AAV-B19- $\gamma$ -globin.

14. The vector of Claim 5 wherein said vector is  
AAV-B19-mdr.

20          15. The vector of any of Claims 1-14 wherein  
said vector is contained in a host cell.

16. The vector of any one of Claims 1-12 wherein  
said vector is contained in a virion.

25          17. The vector of Claim 16 wherein said vector  
is contained in a host cell.

18. The vector of Claim 15 or 17 wherein said  
host cell is a hematopoietic stem or progenitor cell.

19. A method of use of the vector of Claim 1 for  
gene therapy which comprises:

30          a) obtaining bone marrow cells from a patient;  
              b) transducing said bone marrow cells with the  
vector of Claim 1; and

-55-

1           c) reintroducing said transduced bone marrow  
cells into said patient.

20.   The method of Claim 19 wherein said  
transducing is achieved by transfection with said vector  
5   or by infection with virions containing said vector.

22.   The method of Claim 19 wherein said gene  
therapy comprises treatment of sickle-cell anemia or  
diabetes.

23.   The method of Claim 19 wherein said gene  
10   therapy comprises treatment of thalassemia.

24.   The method of Claim 23 wherein said vector  
is AAV-B19- $\alpha$ -globin or AAV-B19- $\beta$ -globin.

25.   The method of Claim 19 wherein said gene  
therapy comprises treatment of hematopoietic diseases.

15       26.   The method of Claim 25 wherein said vector  
is AAV-B19-GM-CSF.

27.   The method of Claim 25 wherein said gene  
therapy comprises treatment of cancer.

28.   The method of Claim 27 wherein said vector  
20   is AAV-B19-RB.

29.   The method of Claim 19 wherein said gene  
therapy comprises treatment of hemoglobinopathies.

30       30.   The method of Claim 29 wherein said vector  
is AAV-B19- $\beta$ -globin, AAV-B19- $\alpha$ -globin or AAV-B19- $\gamma$ -  
25   globin.

31.   A method of use of the vector of Claim 1 for  
delivering a pharmaceutical product in a mammal which  
comprises:

30       a) obtaining bone marrow cells from said mammal;  
b) transducing said bone marrow cells with the  
vector of Claim 1 and

1           c) reintroducing said transduced bone marrow  
cells into said mammal.

5           32. The method of Claim 31 wherein said  
transducing is achieved by transfection or by infection  
with virions containing said vector.

10          33. The method of Claim 31 wherein said product  
is  $\gamma$ -globin, insulin, macrophage colony stimulating  
factor, granulocyte colony stimulating factor,  
erythropoietin, tumor necrosis factor, mast cell growth  
factor, any of interleukins 1-11, p53, adenosine  
deaminase or an antisense RNA molecule.

15          34. The method of Claim 31 wherein said product  
is granulocyte macrophage-colony stimulating factor.

15          35. The method of Claim 34 wherein said vector  
is AAV-B19-GM-CSF.

16          36. The method of Claim 31 wherein said product  
is  $\alpha$ -globin.

17          37. The method of Claim 36 wherein said vector  
is AAV-B19- $\alpha$ -globin.

20          38. The method of Claim 31 wherein said product  
is  $\beta$ -globin.

21          39. The method of Claim 38 wherein said vector  
is AAV-B19- $\beta$ -globin.

25          40. The method of Claim 31 wherein said product  
is retinoblastoma.

26          41. The method of Claim 40 wherein said vector  
is AAV-B19-RB.

30          42. A method of use of the vector of Claim 11  
for conferring cell-specific multidrug resistance to a  
patient which comprises:

31           a) obtaining bone marrow cells from a patient;

-57-

1           b) transducing said bone marrow cells with the  
vector of Claim 11; and

5           c) reintroducing said transduced bone marrow  
cells into said patient.

5           43. A method of use of the vector of Claim 14  
for conferring multidrug resistance to erythroid cells  
of a patient which comprises:

10          a) obtaining bone marrow cells from a patient;  
              b) transducing said bone marrow cells with the  
vector of Claim 14; and  
              c) reintroducing said transduced bone marrow  
cells into said patient.

15          44. The method of Claim 42 or 43 wherein said  
transducing is achieved by transfection with said vector  
or by infection with virions containing said vector.

15          45. The method of any one of Claims 19, 31, 42  
or 43 wherein said bone marrow cells are enriched for  
stem cells before transduction.

20

25

30

35

**SUBSTITUTE SHEET**

1 / 20

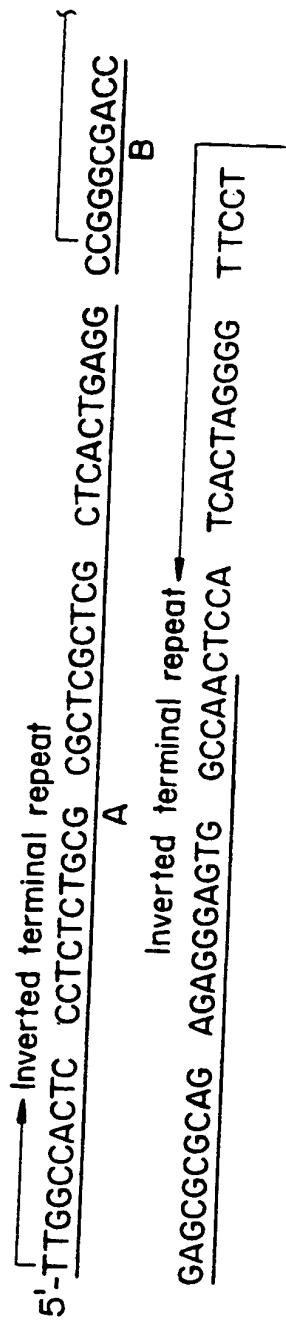


FIG. I

2 / 20

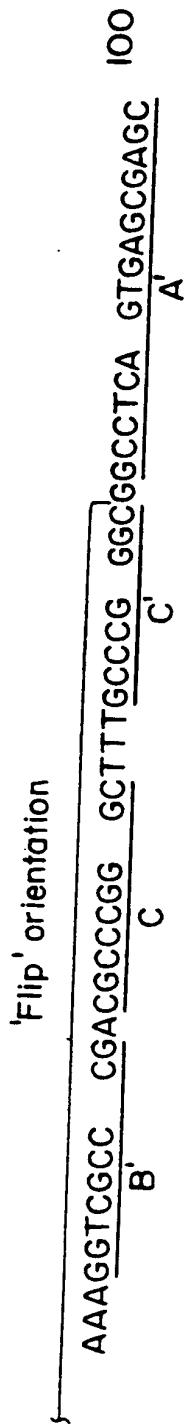


FIG. I CONT.

## **SUBSTITUTE SHEET**

3 / 20

ATTTTAAGTGGTTTACTATAATTTTATTGGTTAGTTTGTAACGGTTAAAATGGGGAGCGTAGGGGGGA  
TAAATTCAACAAATGATATTAAAACATTGCCAATTACCCGCCCTCGCATTCCGCCCT  
EcoRI \* MspI

GCTTTTCCCTGGACTTTCTTGCTGTTTTGTGAGCTAACAGGTATTATACTACTTGT  
CGAAAAGGACCTGAAAGAACGACAAAAAACACTCGATTGATTGTCCATAAATAATGATGAAACAA  
BstNI AluI HpaI  
SrfI

FIG. 2

SUBSTITUTE SHEET

4 / 20

220                    240  
TTTTAGGGCTTTTCCGCCTTATGCCAAATGGGCAGCC  
AAAATCGCCCCGAAAGGGCAGAATACGTTACCCGTCGG  
                        BbvI

320                    340  
CTACAGTATAATAGCACGGTACTGCCAGCTTTCTGGCT  
GATGTCAATAATCGCATGACGGCGTCGAGAAAGACCGA  
                        RsaI      BbvI

FIG. 2 CONT.

5 / 20

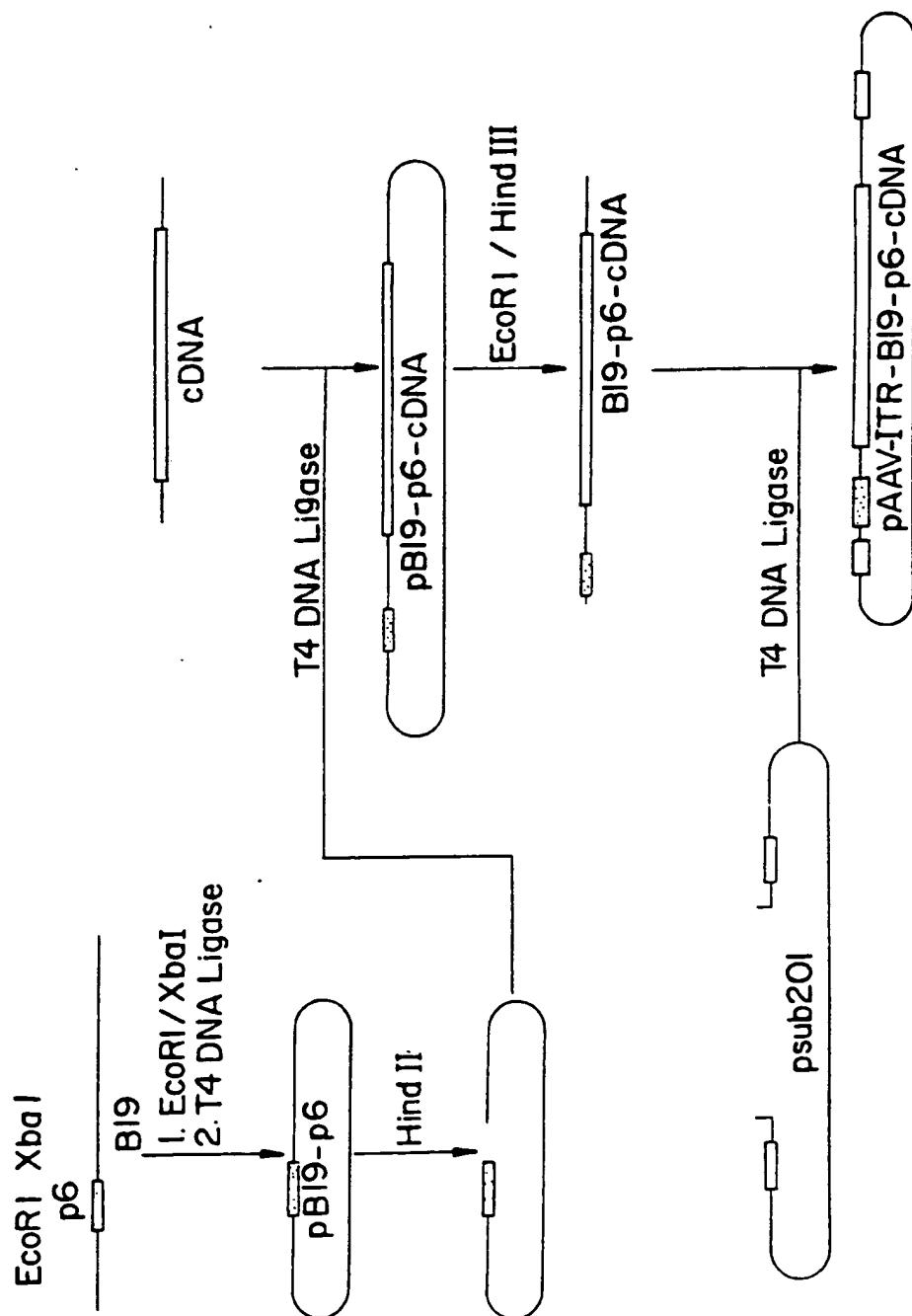


FIG. 3

6 / 20

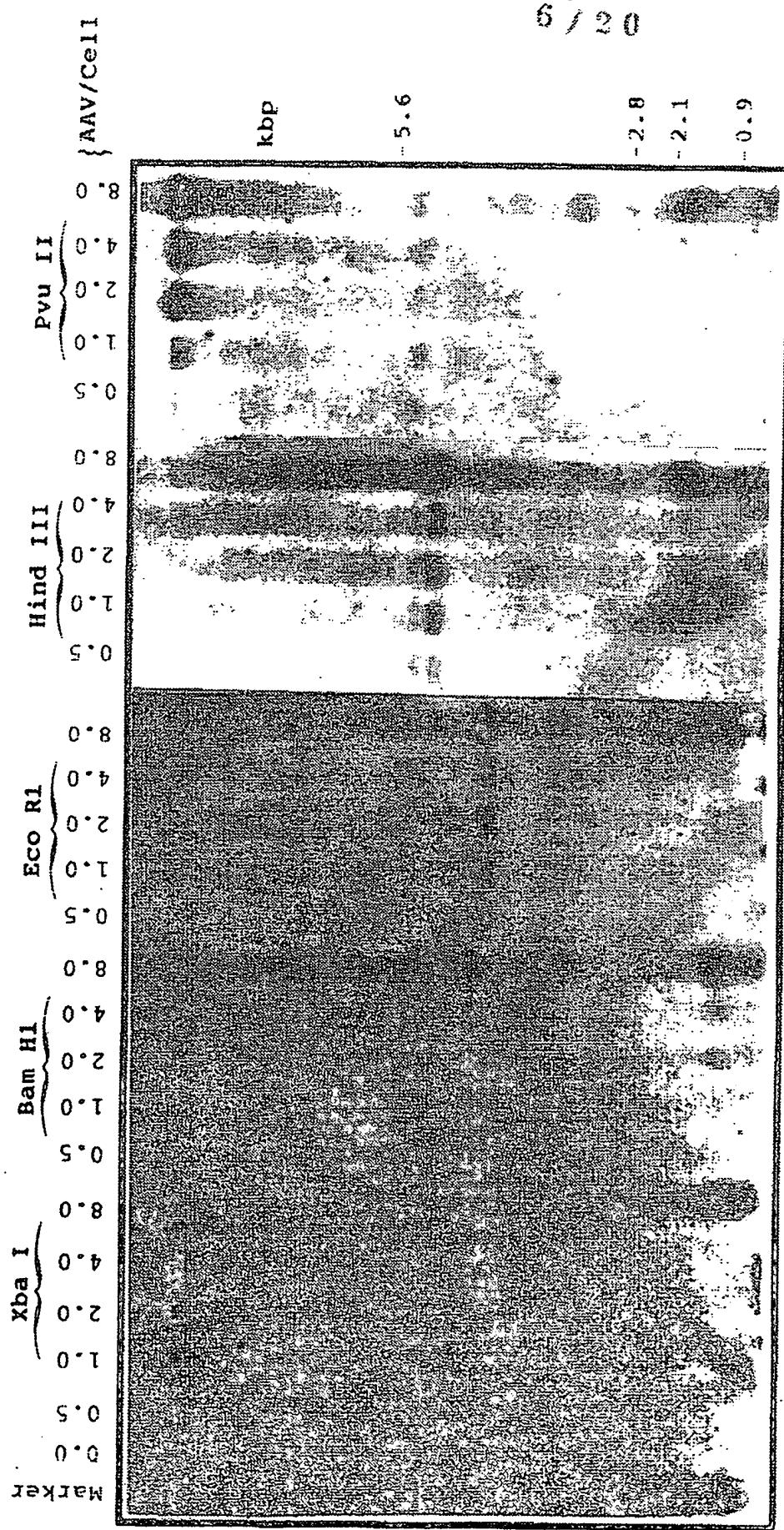
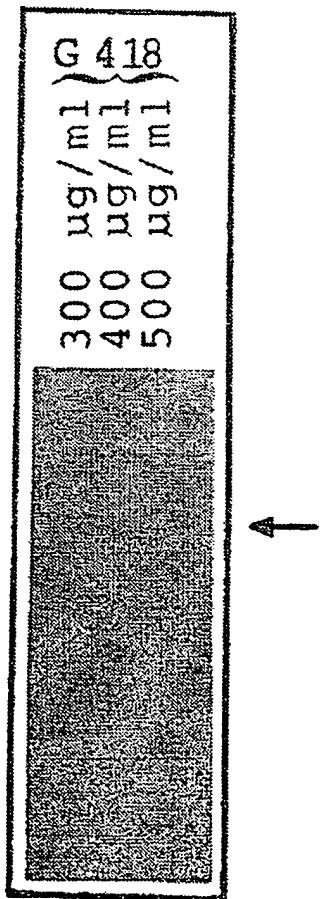


FIG. 4

## **SUBSTITUTE SHEET**

7 / 20

FIG. 5

**SUBSTITUTE SHEET**

8 / 20

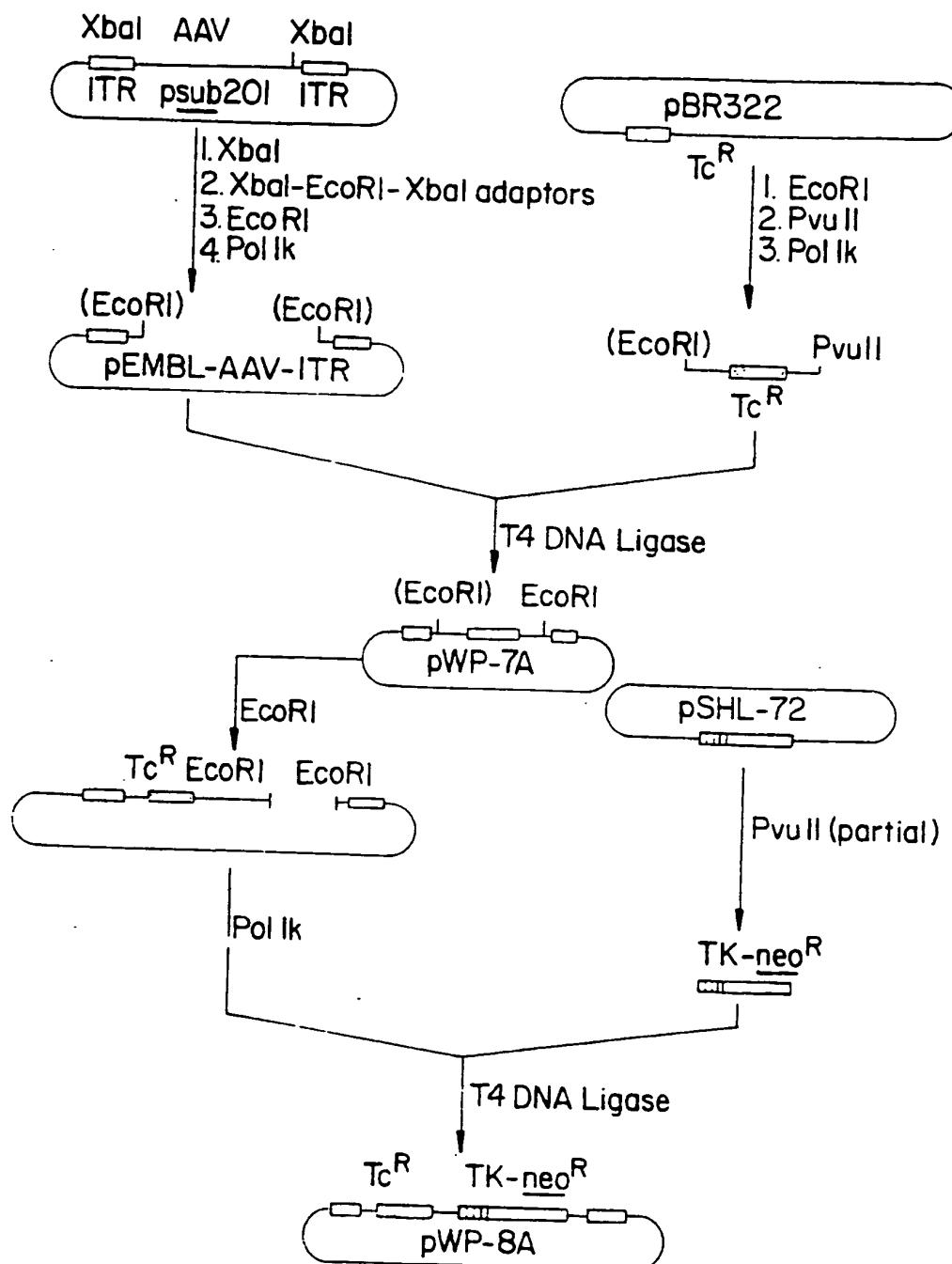


FIG. 6A

9 / 20

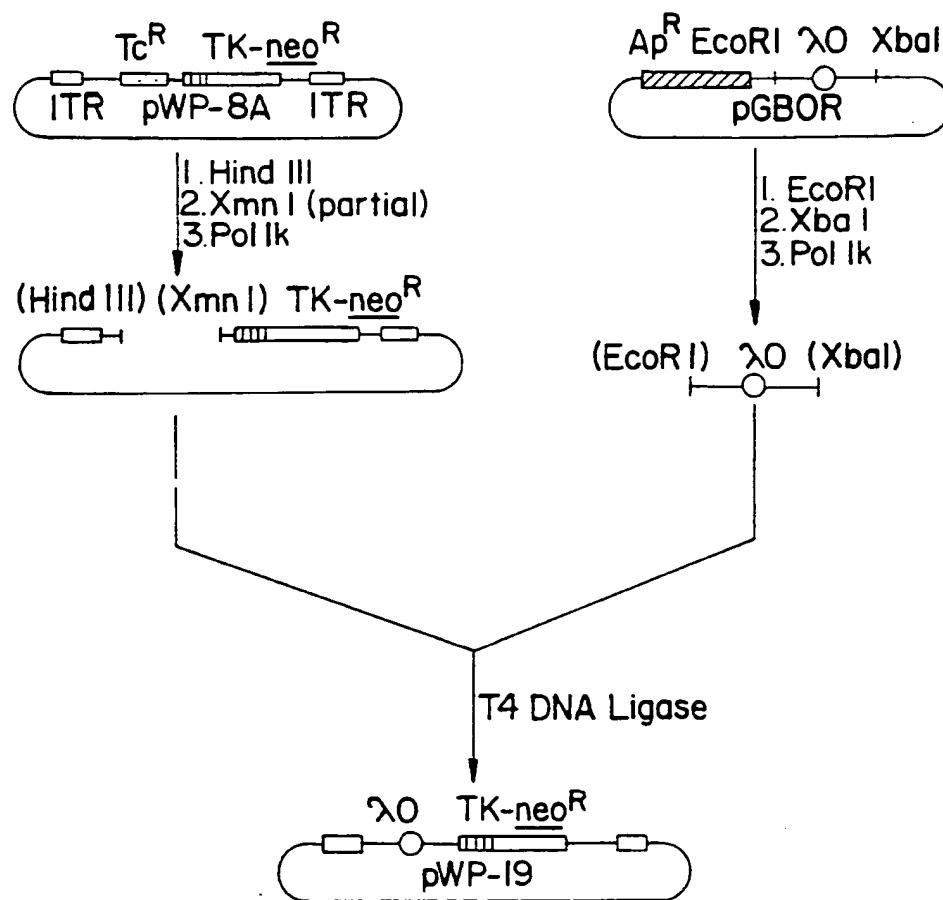


FIG. 6B

10 / 20

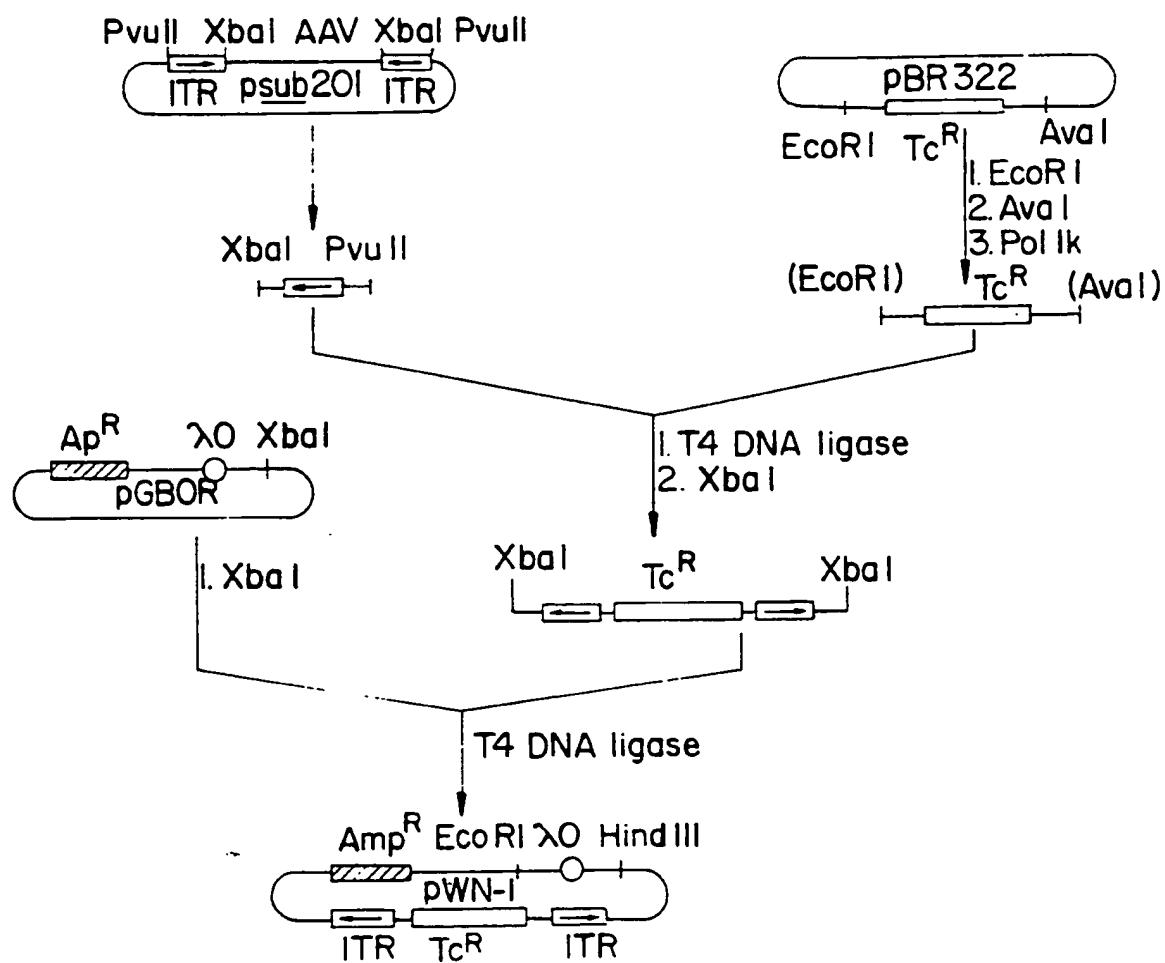


FIG. 7

11 / 20

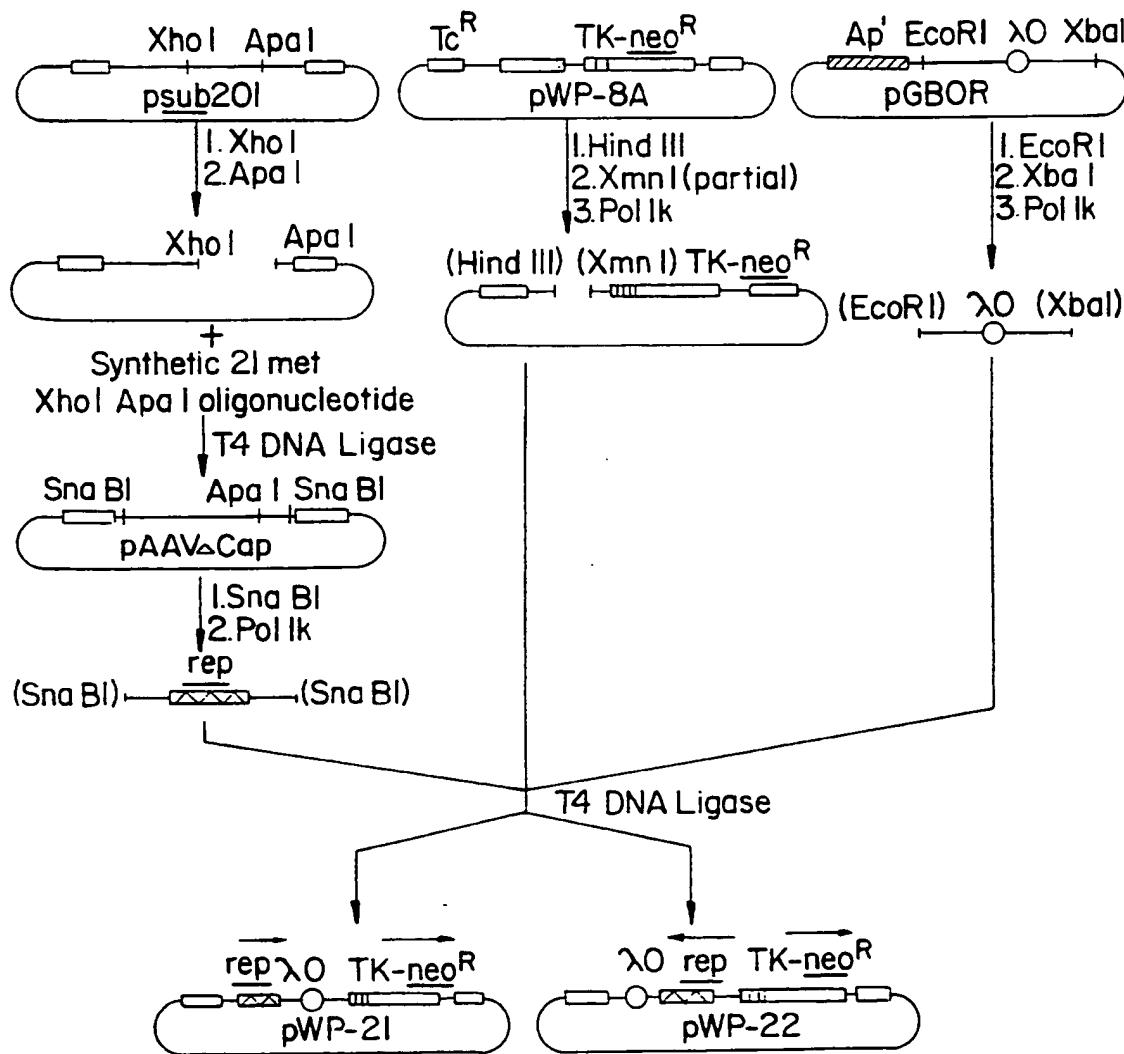


FIG. 8A

**SUBSTITUTE SHEET**

12 / 20

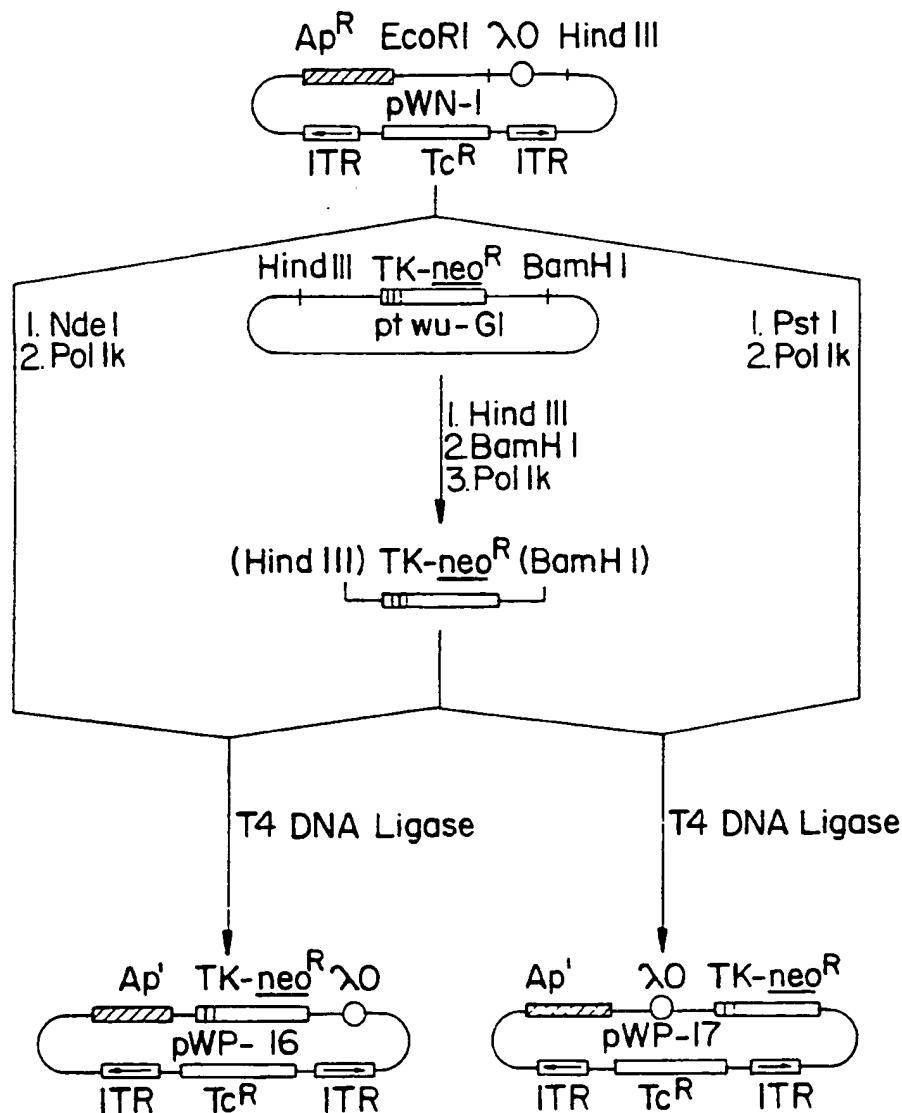
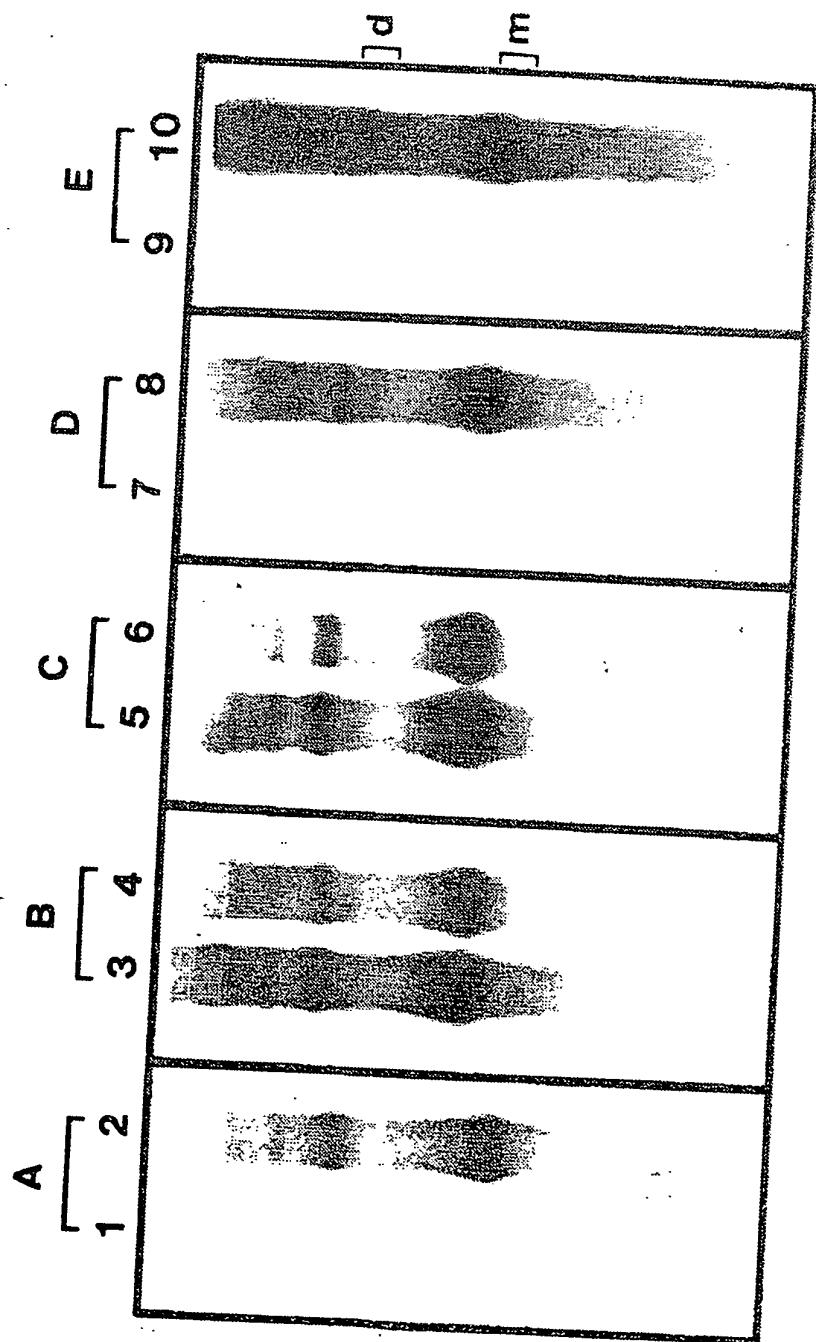


FIG. 8B

13 / 20

FIG. 9

**SUBSTITUTE SHEET**

14 / 20

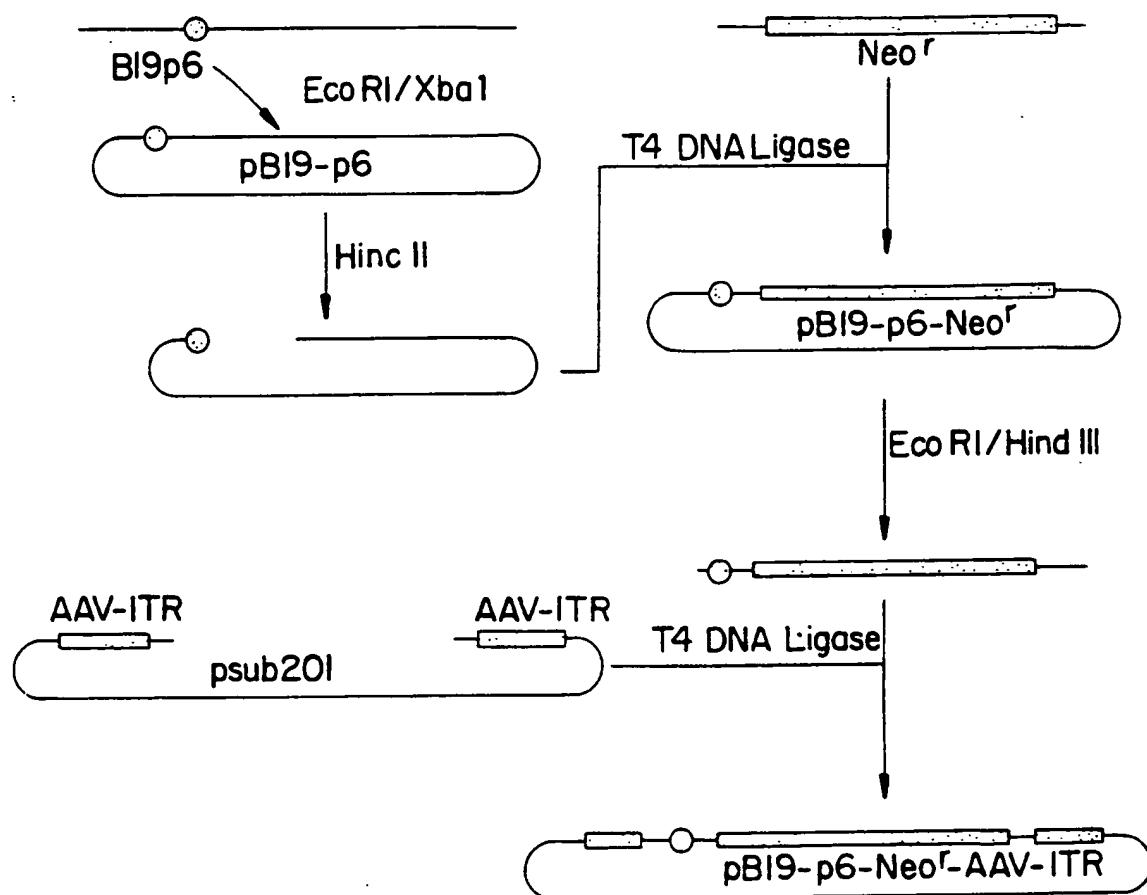


FIG. 10

SUBSTITUTE SHEET

15 / 20

AAV-mediated transfer of Neo<sup>r</sup> to  
human hematopoietic stem cells.

A. Neo gene expression under the  
control of the TK promoter

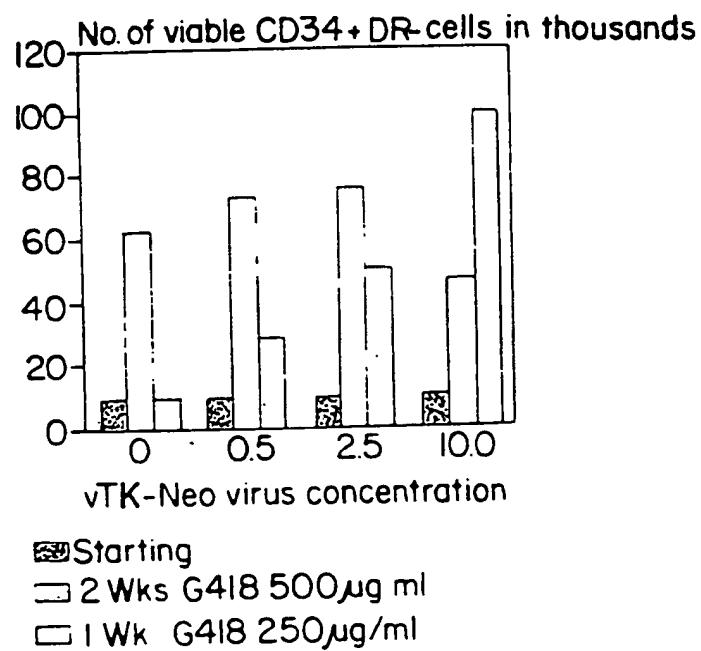


FIG. II A

SUBSTITUTE SHEET

16 / 20

AAV-mediated transfer of Neo to  
human hematopoietic stem cells.

B. Neo gene expression under the  
control of the BI9p6 promotor

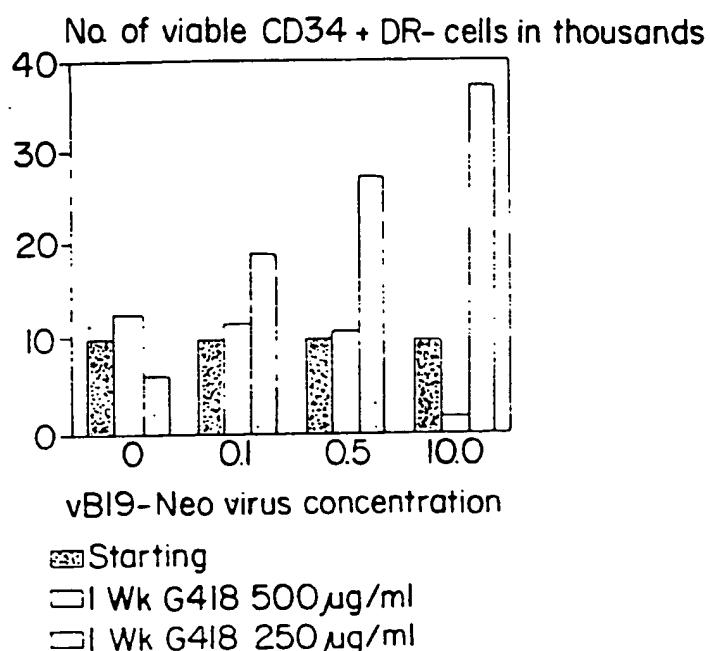


FIG. II B

17 / 20

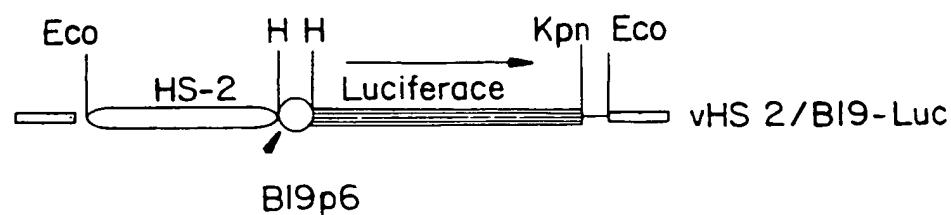


FIG. 12

**SUBSTITUTE SHEET**

18 / 20

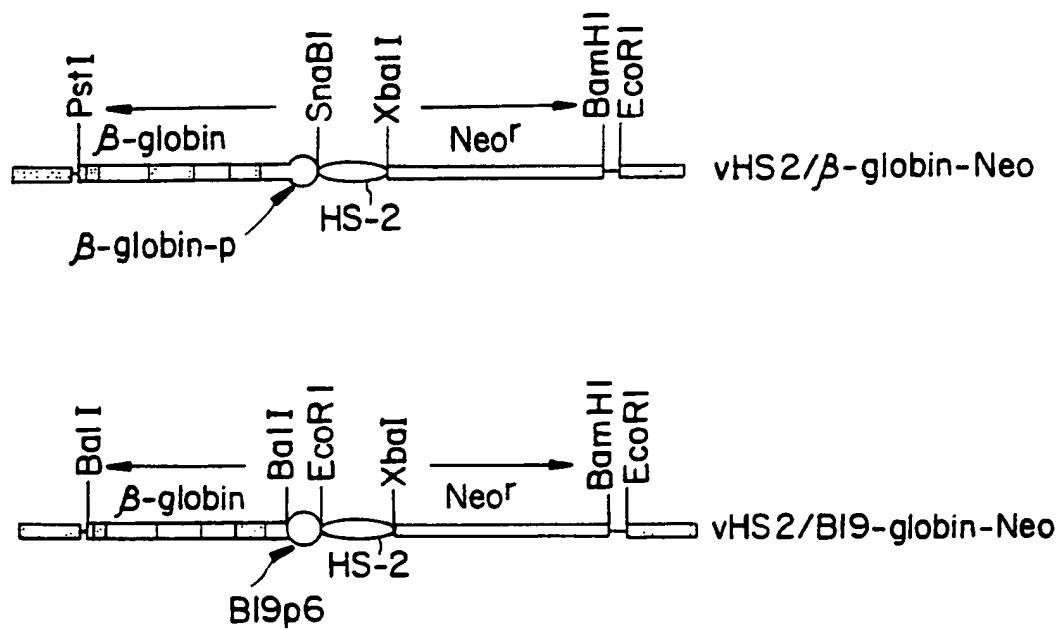


FIG. 13

FIG / 2 n

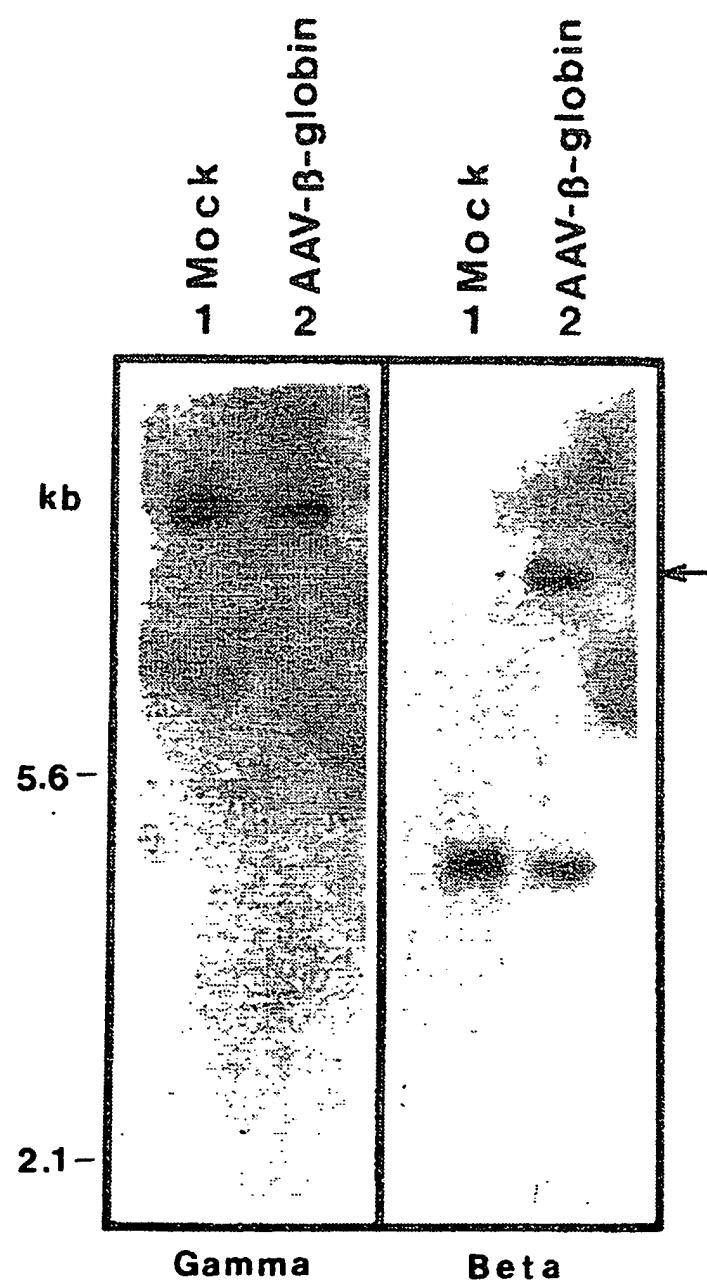


FIG. 14

SUBSTITUTE SHEET

20 / 20

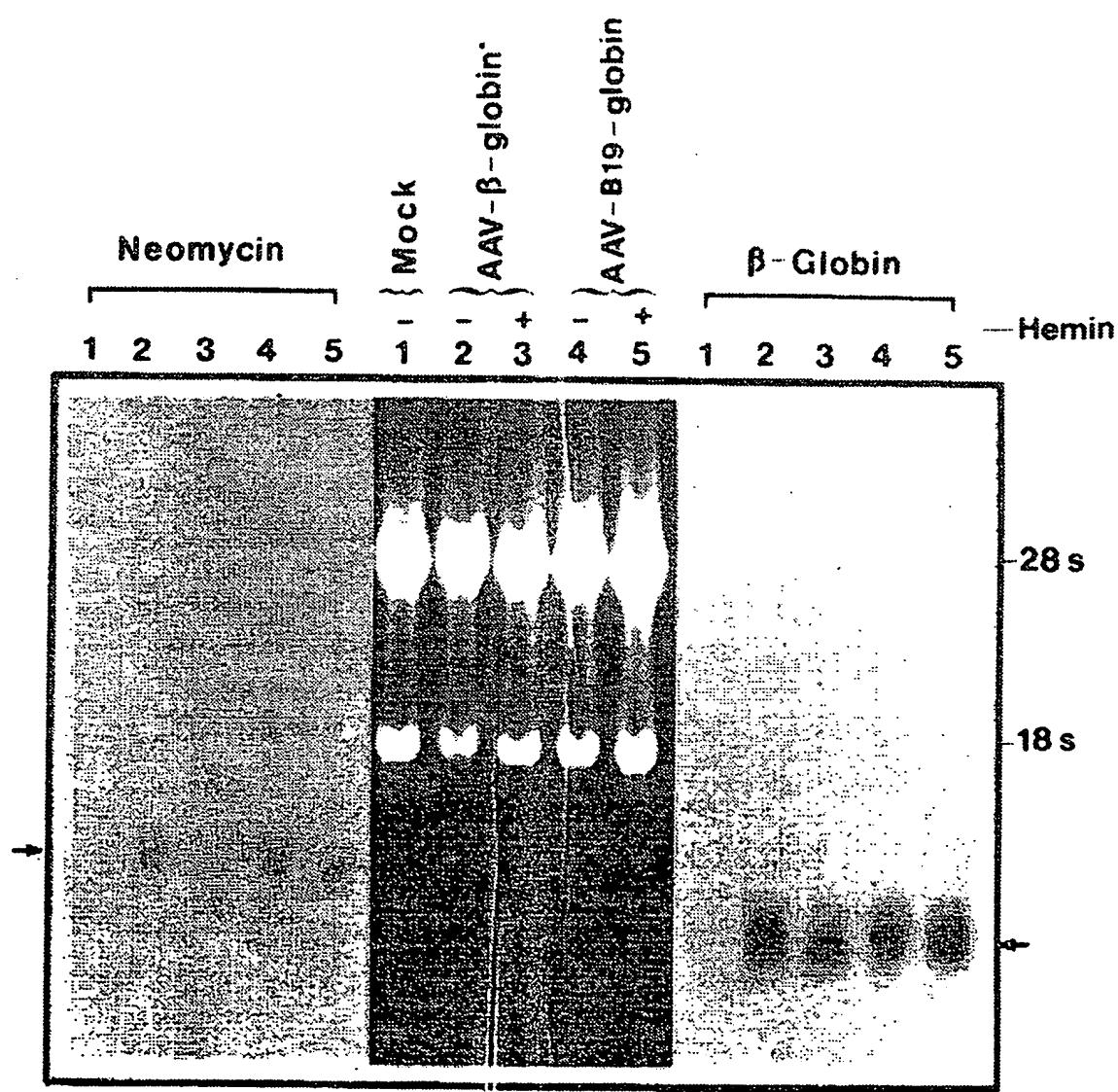


FIG. 15

**SUBSTITUTE SHEET**

## INTERNATIONAL SEARCH REPORT

PCT/US 92/09769

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC  
 Int.Cl. 5 C12N15/86; C12N15/12; A61K48/00

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>

III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	<p>WO,A,8 808 450 (FINLAYSON ,PECK)    3 November 1988</p> <p>see page 5, line 5 - page 7, line 32;    claims 1-89; figure 1</p> <p>see page 34, line 15 - page 36, line 25</p> <p>see page 52, line 22 - page 59, line 10</p> <p>-----</p> <p style="text-align: right;">-/-</p>	<p>1-3, 7,    10, 15,    18-20,    31, 32, 42</p>

<sup>10</sup> Special categories of cited documents :<sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

<sup>11</sup> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<sup>12</sup> "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step<sup>13</sup> "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.<sup>14</sup> "A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search  15 FEBRUARY 1993	Date of Mailing of this International Search Report  05.03.93
---	---

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

HORNIG H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category <sup>a</sup>	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>GENE vol. 89, no. 2, 14 May 1990, ELSEVIER, AMSTERDAM, NL; pages 279 - 282 S. OHI ET AL. 'Construction and replication of an adeno-associated virus expression vector that contains human beta-globin cDNA' see page 279, line 1 - line 8 see page 279, left column, line 1 - page 280, left column, line 2 ---</p>	1-3, 7, 10, 15
Y	<p>VACCINES vol. 90, 1990, CSH LABORATORY PRESS, NEW YORK, US; pages 353 - 359 K.A. VINCENT ET AL. 'Replication and packaging of HIV envelope genes in a novel adeno-associated virus vector system' see page 353, line 1 - line 25 ---</p>	19-45
X	<p>PROC. NATL. ACAD. SCI. vol. 86, no. 20, October 1989, NATL. ACAD SCI., WASHINGTON, DC, US; pages 8078 - 8082 C.H. SRIVASTAVA ET AL. 'Construction of a recombinant human parvovirus B19: Adeno-associated virus 2 (AAV) DNA inverted terminal repeats are functional in an AAV-B19 hybrid virus' cited in the application see page 8078, left column, line 20 - line 22 see page 8081, right column, line 39 - line 45 ---</p>	1-3, 15-17
Y	<p>BLOOD vol. 76, no. 10, 15 November 1990, SAUNDERS, NEW YORK, US; pages 1997 - 2004 A. SRIVASTAVA ET AL. 'Parvovirus B19-induced perturbation of human megacaryocytopoiesis in vitro' cited in the application see page 1997, left column, line 1 - right column, line 15 ---</p>	1-11, 19-45
P,X	<p>WO,A,9 118 088 (THE UNITED STATES OF AMERICA) 28 November 1991 see page 14, line 1 - page 23, line 28 -----</p>	1-3, 7-10, 15

## INTERNATIONAL SEARCH REPORT

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark: Although claims 19-45 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.**
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

## Remark on Protest

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9209769  
SA 67258

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 15/02/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8808450	03-11-88	None	
WO-A-9118088	28-11-91	AU-A- 7906691	10-12-91